Bacterial Degradation of Biarylethers

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SUMMARY

Chlorinated biarylethers such as chlorinated dibenzofurans and dibenzo-*p*-dioxins are environmental contaminants and widely distributed in nature because they resist microbial degradation, which is caused not only by the halogen substituents but also by the extremely stable biarylether linkage. There is enormous interest in microorganisms expressing angular dioxyenases which are indespensible for biarylether degradation. Two bacterial strains capable of utilizing dibenzofuran as a sole carbon source designated HA01 and HA02 were isolated in this study from Egyptian soil and identified as *Rhodococcus* sp. and *Paenibacillus* sp., respectively, on the basis of their 16S ribosomal DNA sequences. *Rhodococcus* sp. strain HA01 was selected for further studies and analyses, as so far no *Rhodococcus* strains capable to mineralize DBF had been described. HA01 was capable to mineralize dibenzofuran, and to transform dibenzo-*p*-dioxin via initial angular dioxygenation albeit with low activity. Also 3-chlorodibenzofuran was transformed mainly by angular dioxygenation with 4-chlorosalicylate as end-product, however lateral dioxygenation occurred also to a minor extend. In contrast, 2-chlorodibenzofuran was transformed by similar extends via angular dioxygenation with 5-chlorosalicylate as product and by lateral dioxygenation giving 2-chloro-3,4-dihydro-3,4-dihydroxydibenzofuran as novel product.

Two gene clusters for the angular dioxygenation of dibenzofuran were isolated and expression during growth on dibenzofuran of both of these gene clusters was confirmed by RT PCR. The *dfdA1A2A3A4* cluster encoded α and β subunits of the terminal oxygenase, ferredoxin, and ferredoxin reductase of $DfdA_{HA01}$ dibenzofuran dioxygenase with high similarity to DfdA dibenzofuran dioxygenase from *Terrabacter* sp. strain YK3. Expression in *Rhodococcus* sp. ATCC 12674 showed $DfdA_{HA01}$ to transform dibenzofuran and 3-chlorodibenzofuran exclusively by angular dioxygenation whereas dibenzo-*p*-dioxin was subject mainly to angular dioxygenation. However, 2-chlorodibenzofuran was not transformed at a significant rate by $DfdA_{HA01}$. A second *dbfA1A2* gene cluster encoded the α and β subunits of the terminal oxygenase of $DbfA_{HA01}$ dibenzofuran dioxygenase with high similarity to DbfA dibenzofuran dioxygenase from *Terrabacter* sp. strain DBF63. Expression in *E. coli* JM109 showed complementary activity of this protein with angular dioxygenase activity against 2-chlorodibenzofuran (as well as dibenzofuran and dibenzo-*p*-dioxin) but not against 3-chlorodibenzofuran. Overall, activities observed in the wild-type strain can be explained by the combined action of both angular dioxygenases and a lateral dioxygenase, revealing that studies wild-type organisms to analyze substrate specificities of angular dioxygenases have to be considered with care.

Also extradiol dioxygenases play a key role in the degradation of dibenzofuran and dibenzo-*p*-dioxin. Using knock-out mutants it could be proven that DbfB extradiol dioxygenase of *Sphingomonas wittichii* RW1, previously reported as involved in dibenzofuran and dibenzo-p-dioxin degradation, is not indispensable for growth but could be substituted by a thus far unidentified extradiol dioxygenase. A detailed kinetic analysis of four extradiol dioxygenases of RW1 (DbfB, Edo2, Edo3 and Edo4) revealed all of them to be subject to severe mechanism based inactivation by 2,2`,3-trihydroxybiphenylether (THBE) the intermediate of dibenzo-p-dioxin degradation with Edo4 being superior as reflected by the relatively high partition ratio and the comparably low efficiency of inactivation, even though Edo4 was evidently not induced during growth on dibenzo-p-dioxin. Significant differences were observed with respect to their inactivation by 3-chlorocatechol and the absence of any significant mechanism-based inactivation makes Edo3 a perfect candidate for being recruited for chlorobiphenyl degradation where inactivation of extradiol dioxygenases by this intermediate creates significant metabolic problems.

ZUSAMMENFASSUNG

Chlorierte Biarylether, wie die chlorierten Dibenzofurane und Dibenzo-*p*-dioxine, sind weitverbreitete Umweltschadstoffe, da sie durch Mikroorganismen schwer abgebaut werden. Diese Resistenz resultiert nicht nur aus der Halogensubstitution, sondern basiert auch auf der hochstabilen Etherbindung. Somit besteht ein großes Interesse an Mikroorganismen, die sogenannte anguläre Dioxygenasen exprimieren, die für den Abbau von Biarylethern unverzichtbar sind. In dieser Arbeit wurden zwei als HA01 und HA02 bezeichnete Bakterienstämme aufgrund ihrer Fähigkeit, Dibenzofuran als einzige Kohlenstoffquelle zu verwerten, isoliert. Die 16S ribosomale DNA-Sequenz identifizierte diese Isolate als *Rhodococcus* sp. und *Paenibacillus* sp. *Rhodococcus* sp. Stamm HA01 wurde für weitere Studien ausgewählt, da bisher keine Dibenzofuran mineralisierenden Rhodococcus Stämme bekannt waren. HA01 mineralisierte nicht nur Dibenzofuran sondern setzte auch Dibenzo-*p*-dioxin durch anguläre Dioxygenierung um, allerdings mit relativ geringer Rate. Auch 3-Chlordibenzofuran wurde überwiegend durch anguläre Dioxygenierung zu 4-Chlorsalicylat als Endprodukt umgesetzt, jedoch wurde in untergeordneter Menge eine laterale Dioxygenierung beobachtet. Im Gegensatz dazu wurde 2-Chlordibenzofuran sowohl durch anguläre Dioxygenierung zu 5-Chlorsalicylat als Endprodukt, als auch in gleichem Ausmaß durch laterale Dioxygenierung zu 2-Chlor-3,4-dihydro-3,4-dihydroxydibenzofuran als neuartigem Produkt umgesetzt.

Während in bisher charakterisierten Dibenzofuran-Abbauern die Anwesenheit nur eines für die anguläre Dioxygenierung verantwortlichen Genclusters beschrieben ist, zeichnete sich HA01 durch zwei solche Cluster aus, die, wie mittels RT-PCR nachgewiesen, beide bei Wachstum mit Dibenzofuran exprimiert werden. Das *dfdA1A2A3A4* Gencluster kodiert für die α und β Untereinheiten der terminalen Oxygenase sowie für Ferredoxin und Ferredoxin Reduktase der $DfdA_{HA01}$ Dibenzofuran Dioxygenase, mit hoher Ähnlichkeit zur DfdA Dibenzofuran Dioxygenase des Stammes *Terrabacter* sp. YK3. Expression in *Rhodococcus* sp. ATCC 12674 zeigte, dass $DfdA_{HA01}$ Dibenzofuran und 3-Chlordibenzofuran exklusiv durch anguläre Dioxygenierung umwandelt, während Dibenzo-*p*-dioxin vorwiegend einer angulären Dioxygenierung unterlag. 2-Chlordibenzofuran wurde durch $DfdA_{HA01}$ nicht umgesetzt. Ein zweites *dbfA1A2* Gencluster kodiert für die α and β Untereinheiten der terminalen Oxygenase der $DbfA_{HA01}$ Dibenzofuran Dioxygenase mit hoher Ähnlichkeit zu derjenigen des Stammes *Terrabacter* sp. DBF63. Expression in *E. coli* JM109 zeigte eine komplementäre Aktivität dieses Proteins, welches 2-Chlordibenzofuran (sowie Dibenzofuran and Dibenzo-p-dioxin) durch anguläre Dioxygenierung umsetzte, jedoch nicht 3-Chlordibenzofuran. Zusammenfassend können die im Wildtyp beobachteten Aktivitäten aus dem Zusammenspiel beider angulärer Dioxygenasen und einer nicht weiter untersuchten lateralen Dioxygenase erklärt werden. Dies zeigt, dass Untersuchungen an Wildtyp Stämmen zur Aufklärung der Substratspezifität angulärer Dioxygenasen mit Vorsicht zu betrachten sind.

Extradiol Dioxygenasen spielen ebenfalls eine Schlüsselrolle beim Abbau von Dibenzofuran und Dibenzo-*p*-dioxin. Mittels knock-out Mutanten konnte gezeigt werden, dass die DbfB Extradiol Dioxygenase des Stammes *Sphingomonas wittichii* RW1, welche als am Abbau von Dibenzofuran and Dibenzo-p-dioxin beteiligt beschrieben wurde, für ein Wachstum verzichtbar ist und durch eine bisher nicht identifizierte Extradiol Dioxygenase ersetzt werden kann. Eine detaillierte kinetische Analyse von vier Extradiol Dioxygenasen des Stammes RW1 (DbfB, Edo2, Edo3 and Edo4) zeigte, dass alle einer signifikanten im Enzymmechanismus begründeten Inaktivierung durch 2,2`,3-Trihydroxybiphenyl Ether, das Zwischenprodukt des Dibenzo-*p*-dioxin Abbaus unterliegen. Edo4 erwies sich, obwohl es beim Wachstum mit Dibenzo-*p*-dioxin nicht induziert ist, als den anderen Enzymen überlegen, was durch das hohe "partition ratio" und die vergleichbar geringe Effizienz der Inaktivierung belegt ist. Drastische Unterschiede wurden bezüglich der Inaktivierung durch 3-Chlorbrenzcatechin beobachtet. Das Fehlen einer signifikanten Inaktivierung zeigt, dass Edo3 ein perfektes Enzym darstellt, um den Abbau von Chlorbiphenylen zu optimieren, welcher durch 3-Chlorbrenzcatechin oft drastisch gestört wird.

ABBREVIATIONS

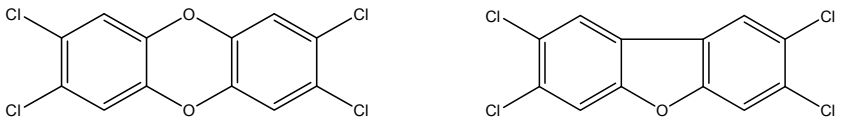
A	*Adenine*
Ap	*Ampicillin*
APS	*Ammonium persulphate*
ATP	*Adenosine triphosphate*
BPH	*Biphenyl*
BSA	*Bovine serum albumin*
bp	*Base pairs*
CAR	*Carbazole*
CBP	*Chlorinated biphenyl*
CDBF	*Chlorinated dibenzofuran*
2CDBF	*2-Chlorodibenzofuran*
3CDBF	*3-Chlorodibenzofuran*
CDD	*Chlorinated dibenzo-p- dioxin*
DBF	*Dibenzofuran*
DD	*Dibenzo-p-dioxin*
DHB	*2,3- Dihydroxybiphenyl*
DMSO	*Dimethyl sulfoxide*
DNA	*Deoxyribonucleic acid*
dNTP	*Deoxynucleotide triphosphate*
FPLC	*Fast Protein liquid Chromatography*
HPLC	*High Performance Liquid Chromatography*
IPTG	*Isopropyl-thio-β -D-galactopyranoside*
kb	*Kilobase*
kDa	*Kilodalton*
Km	*Kanamycin*
NT	*not tested*
OD	*Optical density*
ORF	*Open reading frame*
PAGE	*Polyacrylamide gel electrophoresis*
PAH	*Polycyclic aromatic hydrocarbons*
PCBs	*Polychlorinated Biphenyls*
PCDBFs	*Polychlorinated dibenzofurans*
PCDDs	*Polychlorinated dibenzo-p-dioxins*
PCR	*Polymerase chain reaction*
rpm	*Rounds per minute*
SDS	*Sodium dodecyl sulphate*
TAE	*Tris-acetate/EDTA*
TEMED	*N,N,N,N-Tetramethylethylenediamine*
THB	*2,2',3- trihydroxybiphenyl*
THBE	*2,2',3- trihydroxybiphenyl ether*
Tris	*Tris(hydroxymethyl) aminomethane*
UV	*Ultraviolet*
X-Gal	*5-bromo-4-chloro-3-indolyl-β -D-galactoside*

1 INTRODUCTION

Environmental pollution caused by the release of a wide range of compounds due to industrial progress has now reached severe dimensions. Thousands of hazardous waste sites have been generated worldwide resulting from the accumulation of pollutants in soil and water over the years, often comprising persistent organic chemicals with halo- or nitrosubstituents rarely found in nature.

The halogenated aromatic pollutants include compounds like chlorinated phenoxy herbicides, polychlorinated biphenyls (PCBs) or chlorinated biarylethers. Whereas chlorinated phenoxy herbicides, have been intendedly released into the environment, polychlorinated biphenyls have been manufactured and used widely mainly in closed systems, as heat-transfer fluids, hydraulic lubricants, dielectric fluids for transformers and capacitors, organic diluents, plasticizers, pesticide extenders, adhesives, dust-reducing agents, cutting oils, flame retardants, sealants and in carbonless copy paper (Pieper, 2005). Typical commercially used PCB mixtures contained between 20 and 70 of the 209 theoretically possible congeners and it is estimated that more than 1.5 million tons of PCBs have been manufactured worldwide (De Rosa *et al.*, 2003), where a significant amount has been released into the environment and accumulated in soils and sediments (Nogales *et al.*, 1999; Salata *et al.*, 1995). Biarylethers, comprise several chemical groups such as dibenzo-*p*-dioxins (DDs), dibenzofurans (DBFs) and diphenyl ethers, and their halogenated derivatives, polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDDs/PCDBFs) (see Fig. 1.1). PCDDs/PCDBFs are unintentionally formed as contaminating byproducts during the manufacturing of pesticides, incineration of industrial and domestic wastes, and bleaching of paper pulp.

PCBs as well as PCDDs and PCDBFs have been shown to cause cancer (Mayes *et al.*, 1998) and a number of serious effects on the immune, reproductive, nervous and endocrine system (Albers *et al.*, 1996; Aoki, 2001; Bajanowski *et al.*, 2002; Beck *et al.*, 1994; Bellin & Barnes, 1985; Brewster *et al.*, 1988; Dahl *et al.*, 1995; Ferre-Huguet *et al.*, 2006; Kahn *et al.*, 1988; Karmaus *et al.*, 2005; Kumagai *et al.*, 2002; Otles & Yildiz, 2003) and are among the most problematic environmental pollutants. With eight carbon atoms to be capable to react with chlorine, 135 PCDBF and 75 PCDD congeners are known, and both, the physical and biological properties of each congener are different. The most extensively studied congener of all PCDBFs and PCDDs is 2,3,7,8-TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) (Fig. 1.1), which is also the most potent toxic (McConnell *et al.*, 1978). The toxic potency of other congeners has been graded into toxic equivalent factors (TEFs) based on their relative toxicity compared with 2,3,7,8-TCDD, which was designated as 1 (Safe, 1990).



(TCDD) **(TCDBF)**

Fig. 1.1. Chemical structures of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 2,3,7,8-tetrachlorodibenzofuran (TCDBF).

Because of the toxicity and chemical inertness of dioxins, their removal from polluted environments is one of the most challenging problems in environmental technology. A number of physicochemical techniques for detoxifying and degrading dioxins, such as thermal remediation, photodegradation, and dechlorination with metal catalysts, have been developed and considered for application (Rogers, 1998) However, physicochemical methods are not feasible to remedy large areas of polluted soils and sediments from both ecological and economical viewpoints. Whereas the ultimate goal of remediation is conversion of toxic organic contaminants to simple, less-toxic constituents, by using physicochemical techniques, incomplete conversion can occur and stable intermediates may be formed. Chemical remediation may result in products with increased biological activity. For example, pyrene, a four-ringed polycyclic aromatic hydrocarbon, can be transformed by ozone. This ozonation results in the formation of at least 10 major products, some of which are more mutagenic than pyrene itself (Sasaki J. *et al.*, 1995).

Microorganisms play important roles in the degradation and mineralization of xenobiotic and aromatic compounds in natural environments and such capabilities can be used for the clean up of contaminated environments (bioremediation). Bioremediation is considered as a relatively low-cost technology, which usually has a high public acceptance and can often be carried out on site. Bioremediation has been successfully applied as a biotechnological approach for the treatment of oil spills and sites contaminated with relatively easy to degrade petroleum hydrocarbons (Abd Rahman *et al.*, 2006; Atlas & Bartha, 1992; Brakstad & Bonaunet, 2006; Button *et al.*, 1992; Meintanis *et al.*, 2006; Song *et al.*, 2006). Another example is the bioremediation of the herbicide atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-s-triazine) in soil, where the most efficient methods are considered biostimulation or bioaugmentation (Liu & Suflita, 1993). Biostimulation involves supplementing the contaminated soil to change the physical state of the contaminant, thereby converting it to a bioavailable form (Atlas & Bartha, 1992) such as by the addition of surfactants (Singh *et al.*, 2007; Yu *et al.*, 2007) or supplying a nutritional supplement or cosubstrate to stimulate the population of indigenous bacteria capable of catabolizing the contaminant (Adriaens & Focht, 1990). Bioaugmentation refers to the addition to the soil of microorganisms capable of catabolizing the contaminant (Brodkorb & Legge, 1992). Among others, the effect of bioaugmentation on atrazine removal has been studied in the laboratory and various studies showed an increase in transformation rate (Fadullon *et al.*, 1998; Moran *et al.*, 2006; Rousseaux *et al.*, 2003; Silva *et al.*, 2004; Wenk *et al.*, 1998). Thus, biological methods using microorganisms or microbial consortia capable of pollutant degradation have a great appeal in their potential application for environmental remediation.

Also the biodegradation of DD and DBF and their chlorinated analogues has been studied in soil microcosms and *Sphingomonas wittichii* RW1 (see below) was able to grow in soil amended with DD and DBF (Megharaj *et al.*, 1997) and was capable to mineralize these pollutants. Also Halden et al. (Halden *et al.*, 1999) studied the removal by strain RW1 of DD, DBF and of 2-chlorodibenzo-*p*-dioxin from soil

microcosms. Overall, these studies revealed some capabilities of RW1 to perform degradation under *in-situ* conditions, however, the degradation was severely dependent on preparation of the inoculum (whether the strain was preadapted to the soil conditions), the soil type and the type of pollutant (e.g. biotransformation in soil of 2-chlorodibenzo-*p*-dioxin led to significantly reduced survival). Even though also other studies revealed that bacterial strains can metabolize lower chlorinated dibenzo-*p*-dioxins in model soil systems (Habe *et al.*, 2002a; Habe *et al.*, 2002b) bioaugmentation studies are still characterized by trial-and-error approaches which were often unsuccessful, mainly because of the multivariate nature of the systems involved, be this wastewater treatment plants, bulk soils or the plant rhizosphere, and secondly, because of an incomplete understanding of the bacterial catalytic and survival capacities under conditions of stress and the environmental factors governing those responses.

Studies to understand the interaction between xenobiotics and microorganisms in the environment, which became possible due to advances in the analytical methods to study microbial behavior in populations and communities and which allow cultivation-independent identification of *in situ* key players in environmental remediation, however, have to intersect with studies revealing the responses of single bacteria to changing environmental conditions, but also with efforts on a better understanding of metabolic pathways and their molecular determinants.

1.1 Rieske non-heme iron oxygenases and their role in biodegradation

The bacterial degradation of hydrophobic aromatic pollutants is usually initiated by dioxygenases, which utilize molecular oxygen as a required substrate adding both atoms of O_2 to the aromatic ring. In general, this reaction is the most difficult in the degradation of aromatic compounds, and the addition of hydroxyl groups to the highly stable aromatic ring structure activates the molecule for further oxidation and eventual ring cleavage. The activation of aromatics is usually catalyzed by members of the super family of Rieske non-heme iron oxygenases. To cope with the enormous diversity of aromatic compounds created by diagenesis of organic material, this enzyme family has evolved remarkably broad substrate specificity. Members of this super family are known to overall oxidize hundreds of substrates including linked and fused aromatic, aliphatic olefins, and chlorinated compounds and are distributed among a variety of Gram-negative and Gram-positive bacteria capable of degrading key classes of aromatic pollutants (Gibson & Parales, 2000; Kim & Zylstra, 1999; Lang *et al.*, 2003; Wittich *et al.*, 1992; Zylstra *et al.*, 1997)} Rieske non-heme iron oxygenases are soluble, multicomponent enzyme systems comprising two or three separate proteins, and require oxygen, ferrous iron (Fe^{2+}) and reduced pyridine for catalysis. These enzymes consists of an electron transport chain that channels the electrons from NAD(P)H to the catalytic terminal oxygenase component where substrate transformation take place (Fig. 1.2). The terminal oxygenase component usually contains a Rieske [2Fe-2S]-cluster and a mononuclear iron. To date, several oxygenases have been purified and studied in detail. Some of the most in-depth studies have been carried out with naphthalene dioxygenase (NDO) (Fig. 1.2). All three NDO protein components have been purified (Ensley & Gibson, 1983; Haigler & Gibson, 1990a; Haigler & Gibson,

1990b). The reductase is a monomer of approximately 35 kDa. It contains one molecule of FAD and a plant – type iron-sulfur center, and can accept electrons from either NADH or NADPH (Haigler & Gibson, 1990b; Simon *et al.*, 1993). This iron-sulfur flavoprotein reductase transfers electrons from NADH to the Rieske [2Fe-2S] ferredoxin. The oxygenase consists of α and β subunits in an $\alpha_3\beta_3$ configuration (Kauppi *et al.*, 1998). Each α subunit contains a Rieske [2Fe-2S] center and mono-nuclear Fe^{2+} at the active site. For catalysis, an electron is transferred from the ferredoxin to one of the Rieske centers of the oxygenase and finally to the active site Fe^{2+}. The reduced oxygenase catalyzes the addition of both atoms of O_2 to the substrate to form naphthalene *cis*-(1R,2S)-dihydrodiol. NADH is regenerated through the action of a naphthalene dihydrodiol dehydrogenase, which rearomatizes the *cis*-dihydrodiol to a catechol derivative, which is then subject to ring-cleavage and further degradation. The stereospecific addition of molecular oxygen to an aromatic compound to form a *cis*-dihydrodiol (Fig. 1.2) is the rate-limiting step of the pathway shown and results in very interesting molecules from the standpoint of the synthetic chemist. The regio-and stereospecific oxidation of an unactivated aromatic compound is very difficult to accomplish using conventional chemical techniques, which typically produce an array of byproducts that must be separated and destroyed. Their potential for derivatization through arene functionalities makes *cis*-dihydrodiols valuable synthetic building blocks for the synthesis of biologically important pinitols, conduritols, and acyclic sugars (Hudlicky *et al.*, 1999; Sheldrake, 1992) as well as the drugs indinavir (Buckland *et al.*, 1998) and pancratistain (Hudlicky *et al.*, 1996).

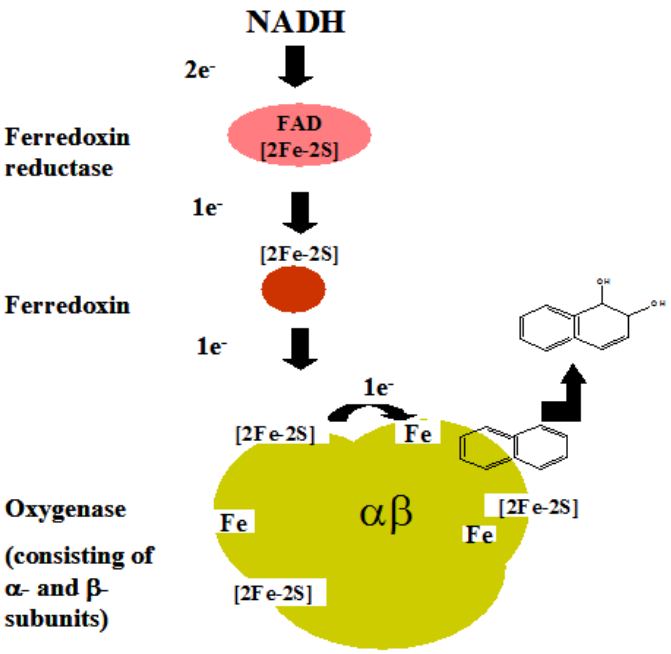


Fig. 1.2. Reaction catalyzed by naphthalene dioxygenase (NDO) (Parales, 2003).

Numerous Rieske non-heme iron oxygenases have been characterized thus far. An early classification system was based on the components of the electron transfer chains present in the Rieske non-heme iron oxygenase systems (Batie *et al.*, 1991). Two-component (reductase, oxygenase) and three-component (reductase, ferredoxin, oxygenase) enzyme systems could be differentiated and these classes were further subdivided based on the number of proteins comprising the oxygenase, the type of flavin moiety (FAD or FMN) present in the reductase, the presence or absence of an iron-sulfur center in the reductase, and the type of iron-sulfur center present in the ferredoxin. This classification system was rather suitable as long as only a small number of enzymes were known. However, with the increasing number and diversity of enzyme systems characterized and the presence of new enzymes with unusual redox partners that do not fit into the original classification it became obvious that such classification was not useful anymore. Moreover, it became clear, that the components of the electron chain are not determining substrate specificity, but was to a certain extent interchangeable between different Rieske non-heme iron oxygenase systems. Werlen et al (Werlen *et al.*, 1996) proposed a classification system based on sequence alignments of the oxygenase α subunits, differentiating four families (naphthalene, toluene/benzene, biphenyl, and benzoate/toluate). Since the oxygenase is the catalytic component and the α subunit plays a major role in determining substrate specificity (Beil *et al.*, 1998; Ju & Parales, 2006; Parales *et al.*, 1998) these classifications are based on the catalytic activity of the enzymes. Further analysis based on α-subunit sequence comparisons confirmed that the grouping of the oxygenases largely correlates with the respective substrate preferences (Gibson & Parales, 2000; Nam *et al.*, 2001). Also Gibson and Parales distinguished four families. Group I, or the phthalate family, comprises Rieske non-heme iron oxygenases that contain only α subunits. Substrates for this diverse group of enzymes include several aromatic acids such as phthalate, *p*-toluate, and phenoxybenzoate, but also carbazole and 2-oxo-1,2-dihydroquinoline. Group II, or the benzoate family represents a cluster of enzymes with activities toward various aromatic acids (see Fig. 1.3). Naphthalene, phenanthrene, and nitroarene dioxygenases clustered as group III and were termed naphthalene family. Biphenyl, toluene and benzene dioxygenases were observed to be highly similar in sequence and thus grouped as one cluster (Group IV or the toluene/biphenyl family). With the increasing interest in microorganisms capable to degrade aromatic pollutants as well as naturally occurring aromatics, however, various enzymes were characterized in the recent years, which were only distantly related to above described oxygenases (Fig. 1.3), among them dioxin dioxygenase from *Sphingomonas wittichii* RW1 (Armengaud & Timmis, 1997) and dibenzofuran dioxygenases from *Terrabacter* sp. strain DBF63 and *Terrabacter* sp. strain YK3 (Iida *et al.*, 2002a; Kasuga *et al.*, 2001), enzyme systems described to be involved in the degradation of biarylethers. This, as well as various ongoing sequencing projects and the culture independent analyses of contaminated ecosystems for the presence of Rieske non-heme iron oxygenases (Witzig *et al.*, 2006) shows, that the natural diversity of such enzymes is by far larger then previously assumed, and new enzymes and evolutionary branches are still being discovered.

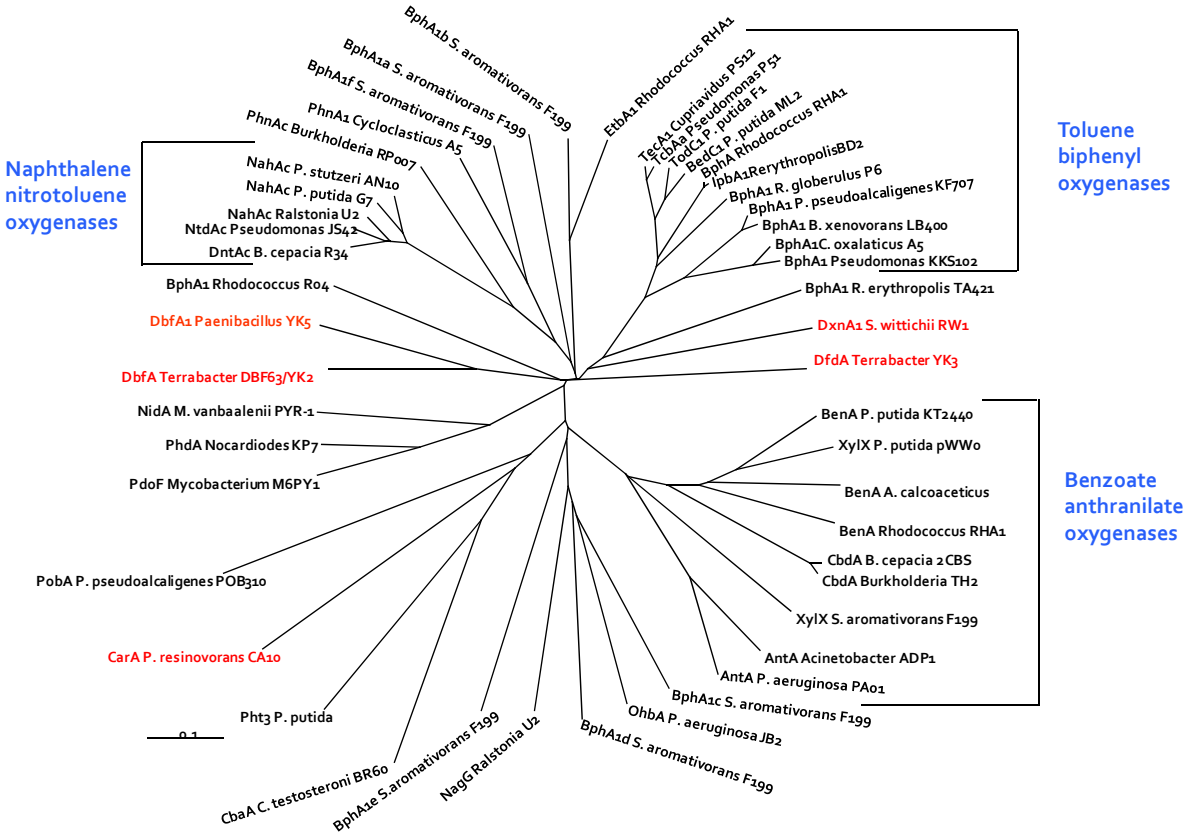


Fig. 1.3. Phylogenetic tree of α subunits of Rieske non-heme iron oxygenases. Members of the benzoate/anthranilate family, the toluene/biphenyl family and the naphthalene/nitrotoluene family are indicated. Oxygenases capable of angular dioxygenation are colored in red.

1.2 Lateral dioxygenation of biarylethers

The bacterial metabolism of naphthalene and biphenyl is known since decades. Biphenyl, by all microorganism characterized so far of being capable to mineralize this compound, is subject to 2,3-dioxygenation, giving, after dehydrogenation, 2,3-dihydroxybiphenyl (Fig. 1.4 A). 2,3-Dihydroxybiphenyl is then subject to extradiol ring cleavage by 2,3-dihydroxybiphenyl 1,2-dioxygenases giving rise to 2-hydroxy-6-phenyl-6-oxohexa-2,4-dieneoate (HOPDA) which is in turn hydrolyzed to benzoate and 2-hydroxypenta-2,4-dienoate (Pieper, 2005). The transformation of DBF and DD (Cerniglia *et al.*, 1979; Klecka & Gibson, 1979; Klecka & Gibson, 1980) using naphthalene or biphenyl degrading *Pseudomonas* or *Sphingomonas* strains indicated that dibenzo-*p*-dioxin (DD), dibenzofuran (DBF) and chlorinated derivatives were transformed into dead-end products. The substrates were attacked, in analogy to the biphenyl or naphthalene transformation, at the lateral 1,2- and 2,3-positions, giving rise to dihydrodiols, which were subsequently dehydrogenated to dihydroxy-compounds, and, in case, subject to ring-cleavage (Fig. 1.4). Whereas such a lateral dioxygenation is appropriate to initiate degradation of biphenyl and naphthalene, various authors have shown that it is inappropriate for the degradation of biarylethers (Bianchi *et al.*, 1997; Resnick & Gibson, 1996; Selifonov, 1992). In case of DD transformation the *cis*-1,2-dihydrodiol was the only oxygenation product formed, which was further dehydrogenated to

the 1,2-diol. No further transformation of this product was observed. DBF was attacked at both the 1,2- and 2,3-positions, which were subsequently dehydrogenated to dihydroxy-compounds, and subject to ring-cleavage (Fig. 1.4B). Most reports have thus far indicated that bacterial degradation of DBF and derivatives via lateral dioxygenation is a cometabolizing process and it was assumed that bacteria having only this type of metabolism are unable to mineralize DBF (Wittich, 1998).

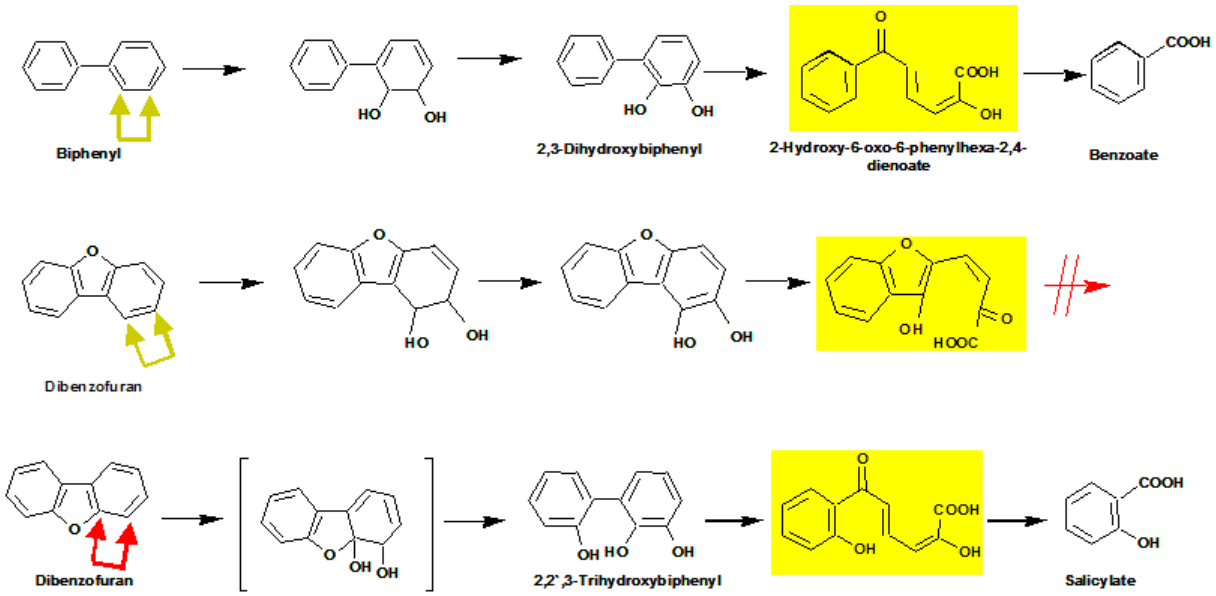


Fig. 1.4. Metabolism of biphenyl via lateral dioxygenation and the biphenyl upper pathway (A), of DBF via lateral dioxygenation (B) and of DBF via angular dioxygenation (C).

1.3 Biodegradation of biarylethers via angular dioxygenation

As transformation of biarylethers by microorganisms capable to degrade naphthalene or biphenyl results in the formation of dead-end products, but none of described organisms could

grow on DBF or DD, it was argued that cleavage of the ether bridge is critical for the degradation of biarylethers. DBF and diphenylethers were then used as a model to study in more detail the degradation of biarylethers. The identification of 1,10-dihydro-1,10-dihydroxyfluoren-9-one as transformation product of fluorene by DBF-grown cells of *Terrabacter* sp. strain DPO1361 (Engesser *et al.*, 1989) indicated a new type of dioxygenase to be present in this strain, attacking polynuclear aromatic systems in an unusual angular position. It was thus proposed that DBF degradation proceeds via initial angular dioxygenation at the 4 and 4a carbon atoms (Fig. 1.5) resulting in the formation of highly unstable hemiacetal, which, after spontaneous cleavage and rearomatization give rise to 2,2´,3-trihydroxybiphenyl (THB). Accumulation of THB from DBF by a mutant of the DBF degrading *Sphingomonas* sp. strain HH69 (Fortnagel *et al.*, 1990) proved angular dioxygenation to be important for cleavage of the ether bridge. Based on the identification of salicylate as central intermediate in DBF

degradation (Fortnagel *et al.*, 1990; Monna *et al.*, 1993; Strubel *et al.*, 1989) it was proposed that THB is subject to *meta* cleavage and subsequent hydrolysis, giving rise to salicylate, analogous to the transformation of 2,3-dihydroxybiphenyl (DHB) to benzoate by biphenyl-degrading bacteria (Fig. 1.4; 1.5). Further degradation of salicylate can then occur either by a salicylate 1-hydroxylase and catechol as intermediate or by a salicylate 5-hydroxylase and gentisate as intermediate. Angular dioxygenation as initial step was also shown to be the basic principle to achieve degradation of other biarylether compounds. As an example, angular dioxygenation of carboxybiphenyl ethers yields an unstable hemiacetal, which spontaneously rearranges to protocatechuate and phenol (Engesser *et al.*, 1990; Wittich *et al.*, 1990). Initial angular dioxygenation of DD followed by spontaneous rearrangement resulted in the formation of 2,2´,3-trihydroxybiphenyl ether (THBE) (Fig. 1.5) (Wittich *et al.*, 1992).

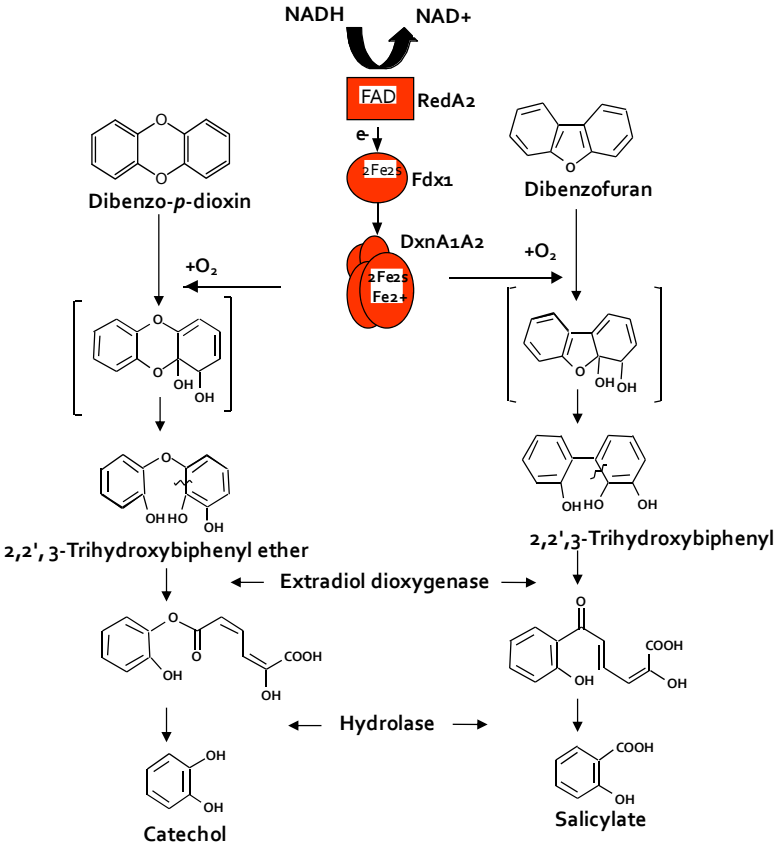


Fig. 1.5. Degradation of DD and DBF by *Sphingomonas wittichii* RW1 and organization of dioxin dioxygenase (RedA2, flavoprotein reductase; Fdx1, ferredoxin; and DxnA1A2, terminal dioxygenase) (Armengaud & Timmis, 1997).

Catechol has been identified as a metabolite further downstream in the DD metabolic pathway and Wittich and coworkers proposed that catechol was formed from THBE after *meta* cleavage (Wittich *et al.*, 1992) Based on the instability of the THBE ring-cleavage product it was speculated, however, that an enzyme catalyzed hydrolysis of the ring-cleavage product is not necessary, and that catechol is formed in a spontaneous reaction. Pfeifer et al. (Pfeifer *et al.*, 1993) reported that *meta* cleavage of DHB ether by 2,3-dihydroxybiphenyl 1,2-dioxygenase of *Pseudomonas cepacia* Et4 leads to the formation of phenol and

2-pyrone-6-carboxylate as products of ring fission indicating that catechol maybe formed spontaneously after ring-cleavage of THBE. However, the spontaneous reaction produces 2-pyrone-6-carboxylate, which, at least in *Pseudomonas cepacia* Et4 is a dead-end product. In contrast, hydrolysis would result in the formation of 2-hydroxymuconate, which is a metabolite of catechol degradation via the *meta*-cleavage pathway and thus an easily degradable metabolite. Thus, it can be proposed that in RW1, which is also not capable to transform 2-pyrone-6-carboxylate, degradation of DD necessitates the activity of an angular dioxygenase, a *meta*-cleavage enzyme and a hydrolase capable to prevent formation of 2-pyrone-6-carboxylate.

For the remediation of dioxin contamination, angular dioxygenation represents an attractive and ideal reaction, as compared to other degradation reactions, since the former reaction forms an unstable hemiacetal intermediate and thus leads to the destruction of the planar structure of dioxin, which determines its toxicity (Nojiri & Omori, 2002). Thus, angular dioxygenases and the bacteria that express them could be important as tools for the remediation of dioxin contamination.

1.4 Biochemical and molecular analysis of biarylether degradation

1.4.1 *Sphingomonas wittichii* RW1

Sphingomonas wittichii RW1 is one of the best-characterized DBF/DD-degrading bacteria and there have been intensive attempts to isolate the enzymes and characterize the genes involved in DBF and DD degradation, since Wittich *et al*. (Wittich *et al.*, 1992) reported the isolation of this strain. Bünz and Cook (Bunz & Cook, 1993) succeeded in purifying the enzyme involved in initial angular dioxygenation from strain RW1 and showed that the dioxygenase belongs to the superfamily of Rieske non-heme iron oxygenases. The enzyme complex (Fig. 1.5) was shown to consist of a terminal oxygenase and electron transfer components. The terminal oxygenase, like that of biphenyl or naphthalene dioxygenases, is a heterodimer composed of a large α subunit and a small β subunit. The electron transfer chain consists of a ferredoxin (Fdx1) of the putidaredoxin-type and a reductase. During purification two isofunctional monomeric flavoreductases were observed, and both were shown to be capable to reduce the ferredoxin involved in electron transport. Genes encoding enzymes of the DD/DBF degradation pathway in RW1 have also been localized. However, in contrast to the common situation of genes encoding enzymes of biphenyl or naphthalene degradation, genes of the dioxin degradation pathway, and even genes encoding for the dioxin dioxygenase subunits were not clustered but assumed to be located on distinct and separate genome segments. Moreover, the α-subunit showed only weak homology to other α-subunits of Rieske non-heme iron oxygenases, 45% identity to the α-subunit of biphenyl dioxygenases from *Rhodococcus globerulus* P6 as nearest neighbor (Asturias & Timmis, 1993), and the electron transport system was unusual in comprising a putidaredoxin-type ferredoxin rather than the usual Rieske-type ferredoxin (Armengaud & Timmis, 1997).

1.4.2 *Terrabacter* sp. strain DBF63

Terrabacter sp. strain DBF63 was isolated from soil based on its capability to utilize DBF and fluorene as a sole source of carbon and energy (Monna *et al.*, 1993). Further studies revealed a catabolic pathway for DBF degradation similar to the one observed in RW1 comprising an angular dioxygenase. The resultant unstable hemiacetal decayed spontaneously to THB, and the dihydroxylated aromatic ring of THB was subject to *meta*-cleavage, followed by hydrolysis of the side-chain to give salicylate (Fig. 1.5) (Kasuga *et al.*, 1997; Kasuga *et al.*, 2001; Monna *et al.*, 1993). Kasuga et al. (Kasuga *et al.*, 2001) could identify the genes encoding the α-and β subunits of the DBF 4,4a-dioxygenase (angular dioxygenase) of DBF63, and expression in *E.coli* of only these subunits resulted in an active enzyme, indicating that ferredoxin and ferredoxin reductase components could be recruited from the *E.coli* host. The DbfA1 α-subunit from strain DBF63 showed only <40% identity with α-subunits of angular dioxin dioxygenase DxnA1 of *Sphingomonas wittichii* RW1 (Armengaud *et al.*, 1998) or DBF dioxygenase, DfdA1 of *Terrabacter* sp. strain YK3 (Iida *et al.*, 2002a) (Fig. 1.3). The highest sequence identity of 60 % is observed with the DbfA1 angular dioxygenase α-subunit of the recently isolated DBF-degrading, *Paenibacillus* sp. strain YK5 (Iida *et al.*, 2006). The *dbfA1* and *dbfA2* genes were revealed to be located within a catabolic gene cluster (Fig. 1.6) (Habe *et al.*, 2004a) comprising genes for phthalate and fluorene degradation.

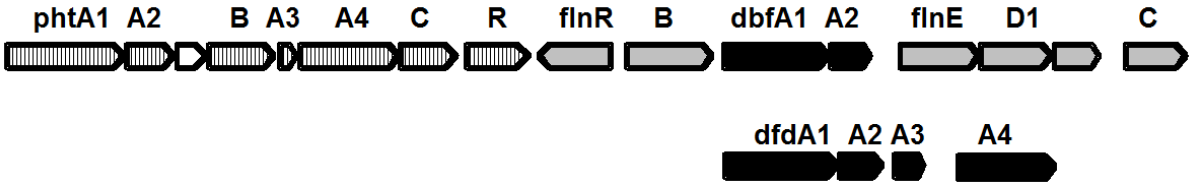


Fig. 1.6. Genetic organization of the loci encompassing the genes involved in DBF, fluorine and phthalate degradation from *Terrabacter* sp. strain DBF63 (Habe *et al.*, 2003; Habe *et al.*, 2004b) (top) and DBF degradation from *Terrabacter* sp. YK3 (bottom) with *phtA1*, phthalate 3,4-dioxygenase large subunit; *phtA2*, phthalate 3,4-dioxygenase small subunit; *phtB*, *cis*-3,4-dihydro-3,4-dihydroxyphthalate dehydrogenase; *phtA3*, phthalate 3,4-dioxygenase ferredoxin; *phtA4*, phthalate 3,4-dioxygenase ferredoxin reductase, *phtC*, 3,4-dihydroxyphthalate decarboxylase, *phtR*, regulator; *flnR*, regulator; *flnB*, 1,1a-dihydroxy-1-hydro-9-fluorenone dehydrogenase; *dbfA1*, dibenzofuran dioxygenase large subunit; *dbfA2*, dibenzofuran dioxygenase small subunit; *flnE*, 2-hydroxy-6-oxo-(2′-carboxyphenl)-hexa-2,4-dienoate hydrolase; *flnD1*, 2′-carboxy-2,3-dihydroxybiphenyl 1,2-dioxygenase; *flnC*, short chain dehydrogenase/reductase; *dfdA1*, dibenzofuran dioxygenase large subunit; *dfdA2*, dibenzofuran dioxygenase small subunit; *dfdA3*, dibenzofuran dioxygenase ferredoxin; *dfdA4*, dibenzofuran dioxygenase ferredoxin reductase.

1.4.3 *Terrabacter* (*Janibacter*) sp. strain YK3

Another type of angular dioxygenase gene cluster (*dbfA1A2A3A4*) was cloned from plasmid pYK3 of the DBF-utilizing *Terrabacter* sp. strain YK3 (Iida *et al.*, 2002a). Phylogenetic analysis of the deduced proteins revealed that both terminal oxygenase components (*dbfA1* and *dbfA2*) were again only distantly related to previously described proteins and formed new branches in the phylogeny (Fig. 1.3). In contrast to the situation observed in RW1 and DBF63, genes encoding all components of a functional angular dioxygenase (α subunit DbfA1, β subunit DbfA2, ferredoxin DbfA3, and ferredoxin reductase DbfA4) were organized in one gene cluster. When *dbfA1* to *dbfA4* were introduced into a non-DBF-degrading

mutant of *Rhodococcus* sp. strain YK2, which had spontaneously lost the plasmid, the ability to degrade DBF was restored in this strain (Iida *et al.*, 2002a). Analysis of the breakdown products indicated that DfdA has angular dioxygenase activity (Iida *et al.*, 2002a). This *dfdA* transformant degraded several aromatic compounds, indicating that the novel angular dioxygenase possesses broad substrate specificity (Iida *et al.*, 2002a).

1.5 Biochemical and molecular analysis of carbazole degradation

Carbazole is a *N*-heterocyclic aromatic compound present in creosote, crude oil, and shale oil (Mueller, 1989; Mushrush, 1999). Carbazole is known to be both mutagenic and toxic (Arcos & Argus., 1968) but has been used as an industrial raw material for the production of dyes, medicines, and plastics (Benedik *et al.*, 1998). Various carbazole-degrading bacteria have been isolated recently (Nojiri & Omori, 2002). The carbazole biodegradation pathway of *Pseudomonas resinovorans* CA10 has been elucidated (Ouchiyama *et al.*, 1993). As shown in Fig. 1.7, carbazole is initially dioxygenated at the C9a carbon, which is bonded to the nitrogen atom (termed the angular position), and its adjacent C1 carbon and thus in an angular fashion as described above for the degradation of DD and DBF (Nojiri & Omori, 2002). The angular carbazole 1,9a-dioxygenase (CARDO) consists of a terminal oxygenase and electron transport proteins (Nam *et al.*, 2002; Sato *et al.*, 1997a). The terminal oxygenase of CARDO CarAa is a homotrimeric enzyme that contains one Rieske [2Fe-2S] cluster (Rieske cluster) and one active site iron (Fe^{2+}) per single subunit. Phylogenetic analysis indicated that the amino acid sequence of CarAa shares rather low-level homology (<19%) with other known catalytic subunits of terminal oxygenases (Fig. 1.3). In addition, CarAa differs from above described terminal oxygenases of angular dioxygenases, that consists of both α- and β-subunits with a $\alpha_3\beta_3$ (Fig. 1.2) configuration, by having an α_3 configuration devoid of β-subunits. The electron transport proteins of CARDO, which mediate electron transport from NAD(P)H to CarAa, comprise a ferredoxin (CarAc), which contains one Rieske cluster, and a ferredoxin reductase (CarAd) which contains one FAD and one plant–type (2Fe-2S) cluster (Nam *et al.*, 2002; Sato *et al.*, 1997a). Angular dioxygenation of carbazole yields an unstable *cis*-dihydrodiol (shown in brackets in Fig. 1.7), which spontaneously rearranges to 2'-aminobiphenyl-2,3-diol.

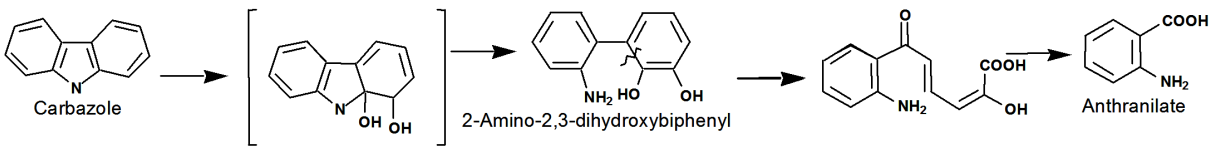


Fig. 1.7. Pathway for degradation of carbazole by *Pseudomonas resinovorans* CA10 which is further degraded by *meta*-cleavage and subsequent hydrolysis to anthranilate (Sato *et al.*, 1997a). This metabolism of carbazole resembles that of DBF and DD, and CARDO was shown to be capable to also catalyze angular dioxygenation of DBF and DD (Nojiri *et al.*, 1999).

1.6 Aerobic biodegradation processes of biarylethers and role of microbial diversity

Over the last decade, a large number of bacterial strains that are capable of degrading biaryl-ether via dioxygenation have been isolated and characterized (Table 1.1). Phylogenetically, almost all of these are belonging to the phyla *Proteobacteria* and *Actinobacteria.* Among members of the phylum Protobacteria, one of the best characterized biarylether degrading bacteria is the alphaproteobacterium, strain RW1, described above, which was isolated from the sediment of the River Elbe, Germany, and identified initially as *Sphingomonas* sp. (Wittich *et al.*, 1992). This bacterium has been proposed as a new species of the genus *Sphingomonas* with the name *Sphingomonas wittichii* (Yabuuchi, 2001).

Table 1.1. Overview of bacteria reported to be capable to degrade DBF, DD, or carbazole and to transform chlorinated dibenzo-p-dioxin or dibenzofurans (CD). G = growth, C = cometabolism, NT= not tested.

Strain	DBF	DD	CAR	CD	Reference
Sphingomonas wittichii RW1	G	G	NT	C	(Arfmann *et al.*, 1997; Armengaud *et al.*, 1999; Bertini *et al.*, 1995; Bunz & Cook, 1993; Bunz *et al.*, 1993; Halden *et al.*, 1999; Happe *et al.*, 1993; Hong *et al.*, 2002; Keim *et al.*, 1999; Megharaj *et al.*, 1997; Wilkes *et al.*, 1996; Yabuuchi, 2001)
Sphingomonas sp. strain HH69	G	C	NT	C	(Fortnagel *et al.*, 1989; Fortnagel *et al.*, 1990; Harms *et al.*, 1990; Harms *et al.*, 1995; Schreiner *et al.*, 1997)
Sphingomonas sp. strain RW16	G	NT	NT	C	(Wittich *et al.*, 1999)
Ralstonia sp. strain RJGII.123	NT	NT	G	NT	(Grosser *et al.*, 1991; Schneider *et al.*, 2000)
Pseudomonas resinovorans CA10	C	C	G	C	(Habe *et al.*, 2001a; Maeda *et al.*, 2003; Nam *et al.*, 2001; Nojiri *et al.*, 1999; Nojiri *et al.*, 2001; Ouchiyama *et al.*, 1993; Sato *et al.*, 1997a; Sato *et al.*, 1997b)
Pseudomonas putida PH-01	G	NT	NT	NT	(Hyo Bong *et al.*, 2000)
Brevibacterium sp. strain DPO220	G	C	C	NT	(Engesser *et al.*, 1989; Strubel *et al.*, 1989)
Terrabacter sp. strain DPO360	G	NT	NT	NT	(Schmid *et al.*, 1997)
Terrabacter sp. strain DBF63	G	C	C	C	(Habe *et al.*, 2001b; Habe *et al.*, 2002b; Kasuga *et al.*, 1997; Kasuga *et al.*, 2001; Monna *et al.*, 1993)
Terrabacter sp. strain YK3	G	C	C	NT	(Iida *et al.*, 2002a)
Janibacter sp. strain YA	G	NT	NT	C	(Iwai *et al.*, 2005)
Janibacter sp. strain XJ-1	G	NT	NT	NT	(Jin *et al.*, 2006)
Janibacter sp. strain YY-1	G	C	C	C	(Yamazoe *et al.*, 2004a)
Paneibacillus sp. strain YK5	G	NT	NT	NT	(Iida *et al.*, 2006)

The family *Sphingomonadaceae* includes a large number of strains, which have been reported to be capable of degrading and assimilating dioxin like compounds (Akira, 2003). The reason why many species of Sphingomonads have the ability to degrade dioxins and related aromatics is not known, but it is of considerable interest whether the hydrophobic and chemical nature (e.g., the presence of glycosphingolipids) of the cell surface of these bacteria is related to the affinity to the aromatic

compounds. Other Gram-negative bacteria reported as biarylether degraders are belonging to the *Gammaproteobacteria* (*Pseudomonas putida* PH01) (Hyo Bong *et al.*, 2000).

Several Gram-positive bacteria with high G+C content, which are belonging to the phylum *Actinobacteria*, (*Terrabacter* and *Janibacter* strains) have been described as biarylether degraders. (Engesser *et al.*, 1989; Habe *et al.*, 2002b; Iida *et al.*, 2002a; Iida *et al.*, 2002b; Kasuga *et al.*, 2001; Kimura & Urushigawa, 2001; Monna *et al.*, 1993; Schmid *et al.*, 1997; Simon *et al.*, 1993; Strubel *et al.*, 1989; Strubel *et al.*, 1991; Trenz *et al.*, 1994). Also *Rhodococcus* is a taxon belonging to the phylum *Actinobacteria*. *Rhodococcus* strains have been reported to be capable to degrade a wide range of aromatic hydrocarbons, and are considered to play a critical role in the biodegradation of toxic pollutants in soil (Larkin *et al.*, 2005). A large set of *Rhodococcus* isolates such as *Rhodococcus* sp. strain RHA1 (Iwasaki *et al.*, 2006; Iwasaki *et al.*, 2007; Kitagawa *et al.*, 2001; Shimizu *et al.*, 2001; Takeda *et al.*, 2004b; Warren *et al.*, 2004), *Rhodococcus* sp. strain T104 (Hernandez *et al.*, 1997; Kim *et al.*, 2002b), *Rhodococcus* sp. strain M5 (Wang *et al.*, 1995), *Rhodococcus globerulus* P6 (Asturias & Timmis, 1993) and *Rhodococcus* sp. strain I24 (Posada, 2006) have been reported to be capable to degrade biphenyl, however, *Rhodococcus* strains capable to degrade DBF via angular dioxygenation had not been reported at the beginning of this thesis, and *Rhodococci* capable to transform DBF did so by lateral dioxygenation resulting in the formation of the dead-end products (Wittich, 1998).

1.7 Biochemical and molecular analysis of the *meta*-cleavage pathway involved in the degradation of biarylethers

The degradation of DBF and DD in all strains capable to mineralize them is initiated by a Rieske non-heme iron oxygenase catalyzing an angular dioxygenation, giving rise to THB or THBE, respectively. These structures already comprise the catecholic structure element necessary to function as substrate for *meta*-cleavage enzymes (extradiol dioxygenases). Thus, in contrast to most aerobic degradation pathways of aromatics involving dioxygenation by Rieske non-heme iron oxygenases, a dehydrogenase, capable of rearomatizing the intermediate dihydrodiol, is not necessary for degradation.

Extradiol dioxygenases are enzymes that mediate aromatic ring cleavage in a wide variety of aromatic pathways, including those of naphthalene, benzene, biphenyl, and toluene (Eltis & Bolin, 1996; Harayama *et al.*, 1992). On the basis of sequence alignments, the extradiol group of dioxygenases has been divided into three families (Vaillancourt *et al.*, 2003). The type II extradiol dioxygenases include enzymes such as protocatechuate 4,5-dioxygenase (LigAB) from *Pseudomonas paucimobilis* (Noda *et al.*, 1990), which has two different types of subunits. The type III extradiol dioxygenases, including 1-hydroxy-2-naphthoate dioxygenase from *Nocardioides* sp. sftrain KP7 (Iwabuchi & Harayama, 1998), belong to the cupin superfamily (Dunwell *et al.*, 2001). The type I extradiol dioxygenases belong to the vicinal oxygen chelate superfamily (Gerlt & Babbitt, 2001). All these extradiol dioxygenases, which cleave the aromatic nucleus adjacent to the hydroxyl substituents (*meta*-cleavage), use non-heme Fe(II) for cleavage (Harayama & Rekik, 1989). However, Mn^{2+}-dependent extradiol dioxygenases with high

sequence similarity to the Fe^{2+}-dependent enzymes have also been reported (Hatta *et al.*, 2003). Harayama and Rekik (Harayama & Rekik, 1989) had proposed that the major type I family of extradiol dioxygenases could be divided into two subfamilies, those showing a preference for monocyclic substrates and those showing a preference for bicyclic substrates (usually determined as the activity with 2,3-dihydroxybiphenyl (DHB) and thus these enzymes are often referred to as 2,3-dihydroxybiphenyl 1,2-dioxygenases). In 1996, Eltis and Bolin (Eltis & Bolin, 1996) analyzed in detail the phylogenetic relationships among type I extradiol dioxygenases and described them as a superfamily which can be divided into different families and subfamilies. Family I.2 and I.3 consist of two-domain iron-containing enzymes that show preferences for monocyclic and bicyclic substrates, respectively, whereas family I.1 comprise the small single domain enzymes identified in *Rhodococcus globerulus* P6 and *Sphingomonas* sp. strain BN6. In the last years, the description of new members of this family increased significantly, due to the sequencing of PCR products amplified from the environment, to genome sequencing projects, but also due to the cloning of the genes from various isolates, specially Gram-positive organisms (Kauffmann *et al.*, 2004) The genes encoding extradiol dioxygenases involved in DBF and DD degradation were usually isolated by shotgun cloning followed by screening of the produced library for expression of activity (Happe *et al.*, 1993; Kasuga *et al.*, 1997; Schmid *et al.*, 1997). This method is straightforward to clone extradiol dioxygenase encoding genes, as the ring cleavage products usually exhibit a bright yellow color, which can be easily identified by eye after spraying of a library with catechol or 2,3-dihydroxybiphenyl. Usually, using this method, various extradiol dioxygenase encoding genes were identified in the genome of the respective organism. Specifically, aromatic hydrocarbon degrading Rhodococci seem to be loaded with such genes (Arai *et al.*, 1998; Asturias & Timmis, 1993; Hauschild *et al.*, 1996; Iida *et al.*, 2002b; Maeda *et al.*, 1995; McKay *et al.*, 2003; Sakai *et al.*, 2002). Five, seven or eight such genes were identified in the genomes of the DBF degrader *Rhodococcus* sp. strain YK2, the biphenyl degraders *Rhodococcus* sp. strain TA421 (Kosono *et al.*, 1997; Maeda *et al.*, 1995), and *Rhodococcus rhodochrous* K37 (Taguchi *et al.*, 2004). Six extradiol dioxygenase genes were identified in *Rhodococcus* sp. strain RHA1 (Sakai *et al.*, 2002), and three are expressed when the organism is grown on biphenyl whereas one of the three genes of *Rhodococcus globerulus* strain P6 (Asturias & Timmis, 1993) was induced during growth on biphenyl and one other was constitutively expressed (McKay *et al.*, 2003). Thus, the presence of multiple extradiol dioxygenases seems to be common in rhodococcal strains and is thought to contribute to the versatility of this group of bacteria in the degradation of haloaromatic compounds. However, despite the huge amount of information on sequence diversity and phylogeny of extradiol dioxygenases, only poor information is available on substrate specificities, specifically for enzymes with high activity against 2,3-dihydroxybiphenyl, which are usually only characterzed by their poor activity with catechol.

Special interest in 2,3-dihydroxybiphenyl 1,2-dioxygenases is due to the fact that various studies indicate that activities of these enzymes limit the degradation of certain chlorinated substrate analogues

(Furukawa *et al.*, 1979; McKay *et al.*, 1997; Seeger *et al.*, 1995). More detailed studies revealed that 2,3-dihydroxybiphenyl 1,2-dioxygenases differ in substrate specificity, but seem to be generally capable of transforming various chlorosubstituted derivatives (Dai *et al.*, 2002; Hein *et al.*, 1998; McKay *et al.*, 2003). However, both 3,4-dihydroxybiphenyl as well as 2′-chlorosubstituted 2,3-dihydroxybiphenyls strongly inhibit 2,3-dihydroxybiphenyl 1,2-dioxygenases (McKay *et al.*, 2003) 2′-Chlorosubstituted 2,3-dihydroxybiphenyls promote suicide inactivation, which involves the release of superoxide during catalysis and oxidation of the active site Fe(II), and thus interfere with the degradation of other compounds (Dai *et al.*, 2002). Beside such similarities in the restricted amount of enzymes tested so far, also significant differences between 2,3-dihydroxybiphenyl 1,2-dioxygenases were observed (Fortin *et al.*, 2005) and specifically single domain extradiol dioxygenases have been shown to be adapted to the transformation of 2′-chlorosubstituted 2,3-dihydroxybiphenyls (McKay *et al.*, 2003).

As described above, despite the abundance of extradiol dioxygenases in various strains, only a small subset is actually involved in the degradation of a given target chemical and the high abundance of extradiol dioxygenases encoding genes possibly reflects the diversity of target chemicals that can be degraded by similar but distinct routes. With respect to degradation of DBF, only one of five 2,3-dihydroxybiphenyl 1,2-dioxygenases observed in *Rhodococcus* sp. strain YK2, *dfdB*, was expressed during growth on DBF (Iida *et al.*, 2002b). An enzyme highly similar (98% sequence identity) to the one encoded by *dfdB* in YK2 and termed BphC1 was also observed to be involved in DBF degradation by *Terrabacter* sp. strain DPO360 (Schmid *et al.*, 1997) and the authors were among the few testing the transformation of THB, which, in contrast to 2,3-dihydroxybiphenyl, is not commercially available. Actually BphC1 behaved like a typical 2,3-dihydroxybiphenyl 1,2-dioxygenase with high activity against 2,3-dihydroxybiphenyl and negligible activity with catechol. Even though 2,3-dihydroxybiphenyl was preferred as substrate over THB, kinetic constants with last mentioned substrate were in accordance with its involvement in DBF degradation. Also the extradiol dioxygenase (DbfB) involved in DBF degradation by *Terrabacter* sp. strain DBF63 (Kasuga *et al.*, 1997) was highly similar to the enzymes of YK2 and DPO360 (94% of sequence identity) (Fig. 1.8) indicating members of this subfamily of extradiol dioxygenases to be of special importance for degradation of DBF.

Fig. 1.8. Dendrogram showing extradiol dioxygenases type I from different bacteria including the three previously described extradiol dioxygenases from *S. wittichii* RW1 (DbfB, Edo2, and Edo3).
The dendrogram was generated using TreeView based on protein sequence alignments calculated by clustalX1.8. The accession numbers are as follows: *Pseudomonas* sp. CA10 (CarB2, D89065); *Pseudomonas* sp. SY5 (BphC1, AF190705); *Rhodococcus* sp. RHA1 (BphC5, AB030672); *Comamonas testosteroni* TA441 (TesB, AB040808); *R. rhodochrous* K37 (BphC4, AB117722); *R. erythropolis* TA421 (BphC1, D88013); *Rhodococcus* sp. RHA1 (BphC2, AB030669); *C. testosteroni* B-356 (BphC, U91936); *R. erythropolis* TA421(BphC5, D88017); *S.wittichii* RW1(Edo2, CP000699); *R .erythropolis* TA421(BphC7, D88019); *R. rhodochrous* K37 (BphC2, AB271916); *P. pseudoalcaligenes* KF707 (BphC, P08695); *Rhodococcus* sp. RHA1 (BphC, D32142); *Burkholderia* sp. TSN101(BphC, BAA97253); *B. xenovorans* LB400 (BphC, P47228); *R. erythropolis* BD2 (IpbC, U24277); *R. rhodochrous* K37 (BphC7, AB271917); *Pseudomonas* sp. JR1 (IpbC, U53507); *R. erythropolis* TA421 (BphC4, D88016); *P. putida* OU83 (BphC, Q52032); *Pseudomonas* sp. Cam-1 (BphC, AY027651); *P. putida* KF715 (BphC, M33813); *R. globerulus* P6 (bphC1, P47213); *M. vanbaalenii* (PhdF, AY365117); *Sphingomonas* sp. CB3 (CarC, AF060489); *P. putida* RE204 (IpbC, AF006691); *Rhodococcus* sp. YK2 (DbfB, AB070453); *Terrabacter* sp. DBF63 (DbfB, AB004563); *S. wittichii* RW1 (DbfB, CP000701); *Terrabacter sp*. DPO360 (BphC, U57649); *P. putida* 01G3 (EbdC, AJ293587); *R. rhodochrous* K37, (BphC1, AB271915), *Nocardioides* sp. KP7 (PhdF, AB031319), *P. putida* F1 (TodE, P13453); *B. japonicum* USDA110 (BphC, BA000040); *Pseudomonas* sp. DJ-12 (PcbC, D44550); *P. azelaica* HBP1 (BphC, U73900); *P. fluorescens* Ip01 (CumC, D37828); *R. rhodochrous* K37 (BphC5, AB117723); *R. palustris* (PsbC2, AB022919), *R. erythropolis* TA421 (bphC6, D88018), *R. rhodochrous* K37; (bphC1, AB271915), *R. pyridinovorans* R04 (BphC2, AY544582); *R. erythropolis* TA431 (BphC, AB272985), *Pseudomonas* sp. SY5, (BphC2, AF190706); *S. yanoikuyae* KF706 (BphC, AB110452); *Ralstonia* sp. SBUG290 (BphC, AJ539227); *R. rhodochrous* K37 (BphC8, AB272984); *P. putida* mt-2 (XylE, P06622); *S. wittichii* RW1 (Edo3, CP000699).

1.8 Biochemical and genetic characterization of extradiol dioxygenases in *Sphingomonas wittichii* RW1

An extradiol dioxygenase involved in the degradation of DBF and DD by RW1 has been described previously (Happe *et al.*, 1993). The enzyme, termed DbfB, exhibited properties similar to those of BphC1 of *Terrabacter* DPO360, with 2,3-dihydroxybiphenyl being preferred over THB as substrate and negligible activity been observed with catechol (Happe *et al.*, 1993). However, phylogenetic analysis indicated DbfB to be only distantly related to any other extradiol dioxygenase described so far (see Fig. 1.8) The enzyme, being constitutively expressed, was shown to be encoded by *dbfB*, localized 4.5 kb upstream of *dxnA1A2* in the RW1 genome and oriented in the opposite direction (Armengaud *et al.*, 1998).

In contrast to 2,3-dihydroxybiphenyl and THB, only poor activity of DbfB was observed with THBE, the intermediate of DD degradation, as a substrate. Moreover, incubation with this substrate resulted in a rapid decline of enzyme activity when measured with 2,3-dihydroxybiphenyl as reporter substrate, indicating a rapid inactivation of the enzyme during THBE transformation (Happe *et al.*, 1993). Since RW1 is able to grow on DD as sole carbon and no major problems during ring-cleavage of intermediates were observed in those growth studies (Wittich *et al.*, 1992), it can be assumed that either DbfB is not inactivated *in vivo*, that inactivation is rapidly overcome *in vivo* by reactivation (ferredoxins such as XylT from *Pseudomonas putida* mt2 have been described to be capable to reactivate extradiol dioxygenases which have been inactivated through reduction of catalytic site oxidized iron, (Hugo *et al.*, 1998)) or that an extradiol dioxygenase distinct from DbfB is actually involved in degradation of DD.

In fact, *Sphingomonas wittichii* RW1 has been shown to contain at least three 2,3-dihydroxybiphenyl 1,2-dioxygenases, with only DbfB having been described in detail (Happe *et al.*, 1993). A second gene encoding a distinct 2,3-dihydroxybiphenyl 1,2- dioxygenase, named Edo2 was briefly described (Armengaud *et al.*, 1998), and encodes a protein with only low sequence similarity to any other known extradiol dioxygenase, sharing 43% amino acid sequence identity with 2,3-dihydroxybiphenyl 1,2-dioxygenase (BpdE) from *Rhodococcus* sp. M5 (Wang *et al.*, 1995) and 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC3) from *Rhodococcus* erythropolis (Kosono *et al.*, 1997). A third gene encoding another distinct 2,3-dihydroxybiphenyl 1,2-dioxygenase, was termed Edo3 (D'Enza, 2002). The encoded protein, in a phylogenetic analysis, showed also only low sequence identity to previously known extradiol dioxygenases (42% identity in the amino acid sequence with 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC1) from *Pseudomonas* sp. SY5 and 41% with CarB2 from *Pseudomonas resinovorans* CA10 (Sato *et al.*, 1997b).

Preliminary analyses (D'Enza, 2002) indicated both Edo2 and Edo3, like DbfB, to be rapidly inactivated during transformation of THBE. It thus still remained to be elucidated, how THBE is metabolized in RW1. Interestingly, the majority of microorganisms capable to mineralize DBF are not capable to grow on DD (Table 1.1) (Iida *et al.*, 2002a; Kasuga *et al.*, 2001; Kubota *et al.*, 2005), indicating some general problems in the degradation of last mentioned compound. As transformation and angular dioxygenation of DD by

DBF degrading organisms is obviously unproblematic and as the capability to degrade catechol is widespread in bacteria, it can be assumed that a major problem in the degradation of DD is the cleavage of THBE.

1.9 Degradation of chlorinated biarylethers

Chlorinated biarylether compounds are highly toxic compounds produced as contaminants during the manufacture of pesticides, pulp and paper or during incinerations. Mineralization of chlorinated dioxins or DBFs by single bacterial strains has not been reported so far. However, different strategies have been followed to allow detoxification of waste containing chlorinated DBFs and dioxins.

It has been known for more than one decade that chloroaromatics can function as an alternative electron acceptor in anaerobic respiration (Mohn & Tiedje, 1990). Several anaerobic Gram-positive bacteria (*Desulfitobacterium* (Sanford *et al.*, 1996) and *Dehalobacter* (Holliger *et al.*, 1998)) and the proteobacteria (*Desulfomonile* (Deweerd & Suflita, 1990) *Desulfuromonas* (Krumholz *et al.*, 1997) and *Sulfospririllum* (Boyle *et al.*, 1999)) have been identified as being able to reductively dehalogenate chlorinated phenols, benzoates, and trichloroethene and to couple this reaction to the synthesis of ATP via a chemiosmotic mechanism (Mohn & Tiedje, 1991). Also PCBs are subject to reductive dechlorination and since the first report on bacterial metabolism of PCBs under anaerobic conditions in sediments of the Hudson River (Brown, 1987) numerous studies have reported on the microbial dechlorination of PCBs *in situ* and in laboratory experiments with sediment slurries. Various dechlorination patterns in environmental and laboratory samples have been described. Typically, *meta* and/or *para* chlorines are removed to generate primarily *ortho*-substituted chlorobiphenyls, but *ortho* dechlorination of several PCB congeners has also been reported (Wiegel & Wu, 2000). This reductive dechlorination of highly chlorinated PCBs decreases their toxicity and increases their aerobic degradability (Bedard & Van Dort, 1997; Mousa *et al.*, 1998; Quensen *et al.*, 1992; Quensen *et al.*, 1998). Organisms mediating the reductive dechlorination of PCBs have been difficult to enrich, and only recently, the respective biocatalysts could be identified, being distantly related to *Dehalococcoides* (Cutter *et al.*, 2001; Wu *et al.*, 2002). Further analysis indicated the presence of dehalogenating systems of different substrate specificity in these organisms. Also the process of reductive dehalogenation of chlorinated DDs is known since more then one decade (Adriaens & Gribic'-Galic', 1994; Adriaens *et al.*, 1995) and the first report of an organism, *Dehalococcoides* sp. strain CBDB1, able to reductively dehalogenate chlorinated DD was published in 2003. Extremely toxic congeners (such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxins) were transformed into di- or monochlorinated congeners (Bunge *et al.*, 2003). Like the case for PCBs, the reductive dehalogenation of chlorinated DDs seems to be restricted to higher chlorinated congeners. However, lower chlorinated PCB congeners can be transformed by aerobic biphenyl degrading organisms (Ahmed & Focht, 1973; Furukawa *et al.*, 1978; Mondello, 1989; Yates & Mondello, 1989). Based on the analysis of various biphenyl degrading isolates it could be deduced that lower chlorinated congeners are more easily transformed compared to higher chlorinated congeners and PCB congeners with chlorines on one aromatic ring only are more easily

degraded than those bearing chlorine substituents on both aromatic rings (Pieper, 2005). However, each isolate exhibited a particular activity spectrum with regard to the type and extent of PCB congeners metabolized, with some strains having a narrow spectrum and others, notably strain LB400 (Mondello, 1989) being able to transform a broad range of congeners.

To a significant extent, the spectrum of PCB congeners that can be transformed by an organism is determined by the specificity of the initial biphenyl 2,3-dioxygenase. Studies on various biphenyl 2,3-dioxygenases have revealed considerable differences in their congener selectivity patterns, as well as their preference of the attacked ring (Erickson & Mondello, 1993; Kimura *et al.*, 1997; McKay *et al.*, 1997; Seeger *et al.*, 1999; Zielinski *et al.*, 2002).

Based on the knowledge gained on transformation of PCBs it can also be assumed that organisms capable to degrade DBF and DD are capable to transform chlorinated derivative. Accordingly, studies have demonstrated the ability of *Sphingomonas wittichii* RW1 not only to degrade unchlorinated DD and DBF but also to transform several mono- and dichlorinated DDs and DBFs (Wilkes *et al.*, 1996) whereas highly chlorinated congeners were recalcitrant. The mono- and dichlorinated DDs and DBFs were transformed to the corresponding mono-and dichlorinated catechols and salicylates, usually together with catechol and salicylate, respectively. These findings indicated that the substituted aromatic ring of the target compounds as well as the non-substituted ones are attacked by angular dioxygenation

Despite its ability to transform mono- and dichlorinated DDs and DBFs, *Sphingomonas wittichii* RW1 is unable to grow with these congeners as carbon and energy sources, due to nonpermissive steps in the degradation pathways for the metabolites produced (Wilkes *et al.*, 1996) and to the best, chlorodibenzofurans were transformed to chlorosalicylates as dead-end products.

The high cometabolic activity of RW1 with 4-chlorodibenzofuran producing 3-chlorosalicylate and the availability of microorganisms capable to mineralize chlorosalicylates, (Schindowski *et al.*, 1991) allowed the establishment of co-cultures which are capable to mineralize the target chemical. In the case of 4-chlorodibenzofuran, dioxygenolytic angular attack by dioxin dioxygenase of strain RW1 took place nearly exclusively at the non-halogenated aromatic ring resulting in stoichiometric production of the dead-end metabolite 3-chlorosalicylate. This metabolite was then utilized as a carbon and energy source by *Burkholderia* sp. strain JWS (Arfmann *et al.*, 1997). In a similar approach, mineralization of 2-chloro- and 3-chlorodibenzofuran was achieved by a co-culture of *Sphingomonas* sp. strain RW16 (which transformed these substrates, beside salicylate, which can be used further by RW16, to 5-chloro- and 4-chlorosalicylate, respectively), with *Pseudomonas moorei* RW10 (capable to mineralize 5-chloro- and 4-chlorosalicylate) (Wittich *et al.*, 1999).

However, transformation of chlorinated DBFs not necessarily results in the formation of chlorosalicylates. The ring-cleavage of THB results in the formation of 2-hydroxy-6-oxo-6-(2-hydroxyphenyl)hexa-2,4-dienoate, which is highly unstable and spontaneously rearranges to form 3-(chroman-4-on-2-yl)-pyruvate(Kohler *et al.*, 1993). During growth on DBF, hydrolases acting on 2-

hydroxy-6-oxo-6-(2-hydroxyphenyl)hexa-2,4-dienoate and transforming it to salicylate and 2-hydroxypenta-2,4-dienoate thus have to compete with this spontaneous reaction. In fact, the accumulation of 3-(chroman-4-on-2-yl)-pyruvate as well as 3-(chroman-4-on-2-yl)-lactate and 3-(chroman-4-on-2-yl)-acetate, among others, during growth of *Sphingomonas* sp. strain HH69 on DBF (Fortnagel *et al.*, 1990) indicated that such a spontaneous rearrangement has taken place and that 3-(chroman-4-on-2-yl)-pyruvate was further processed, possibly by hydrolytic shortening of the side chain. Studies on the catabolism of 2,8-dichloro- (Wilkes *et al.*, 1996), 2,7-dichloro- and 2,4,8-trichlorodibenzofuran (Keim *et al.*, 1999) by *Sphingomonas wittichii* RW1 showed the formation of 6-chloro-and 7-chloro-2-methyl-4H-chromen-4-one, respectively as metabolites, indicating the intermediate di- and trichlorinated 2-hydroxy-6-oxo-6-(2-hydroxyphenyl)hexa-2,4-dienoates to rapidly escape hydrolytic cleavage by rearrangement.

As stated above, also the THBE ring-ceavage product [2-hydroxy-6-oxo-6-(2-hydroxyphenoxy)-hexa-2,4-dienoate] is unstable, and it can be supposed that hydrolysis to give catechol and 2-hydroxymuconate competes with spontaneous rearrangement to give catechol and 2-pyrone-6-carboxylate. However, no data are available on the extend of formation of 2-pyrone-6-carboxylate or chlorinated derivatives during transformation of DD or chlorinated derivatives. Hong et al. (Hong *et al.*, 2002) reported that *Sphingomonas wittichii* RW1 transformed 2,7-DCDD and 1,2,3,4-TCDD to 4-chlorocatechol and 3,4,5,6-tetrachlorocatechol, respectively, however, it remains to be elucidated if such formation is an exclusively enzymatic process.

A thorough analysis on the influence of the substitution pattern on the transformation of PCDD/Fs congeners has been performed by Schreiner et al. (Schreiner *et al.*, 1997). The authors examined the transformation of all 210 PCDD/F congeners by different bacterial strains including *Sphingomonas* sp. strain HH69 harboring a DBF dioxygenase, *Cupriavidus necator* H850 harboring a biphenyl dioxygenase and *Cupriavidus* sp. strain PS12 (Sander *et al.*, 1991) harboring a tetrachlorobenzene dioxygenase. In this experiment, strain HH69 and PS12 were indicated to be the most versatile organisms with HH69 being capable to transform even 2,3,7,8-TCDBF and 2,3,7,8-TCDD.

Above study, like various others, only analyzed substrate depletion and neglected formation of metabolites. However, it is well established that hydroxylated metabolites can exhibit higher toxicity compared to the parent pollutant. As an example, dihydrodiols and dihydroxybiphenyls are very toxic metabolites for bacteria even after short incubation times, affecting the cell viability much more than the parent (chloro) biphenyls (Camara *et al.*, 2004). Taking into account previous analysis that lateral oxygenation of DD results in the formation of dead-end dihydroxylated products (Cerniglia *et al.*, 1979; Klecka & Gibson, 1979; Klecka & Gibson, 1980), it can be questioned if lateral transformation of chlorinated dioxins is actually a detoxification process.

In the case of angular dioxygenation, also problems arising during metabolism are obvious and mechanisms resulting in the formation of 3-(chroman-4-on-2-yl)-pyruvates and 2-pyrone-6-carboxylates

have been discussed above. However, even the formation of catechols from DDs can be deleterious, such as the formation of 3-chlorocatechol from 1-chlorodibenzo-p-dioxin or of 4,5-dichlorocatechol from 2,3-dichlorodibenzo-p-dioxin (Hong *et al.*, 2004; Wilkes *et al.*, 1996). 3-Chlorocatechol is known to be a potent inhibitor of extradiol dioxygenases (Bartels *et al.*, 1984; Wilkes *et al.*, 1996; Wittich *et al.*, 1992) thereby interfering with the upper transformation pathway (Strubel *et al.*, 1991) and 4,5-dichlorocatechol has been reported as strong inhibitor of intradiol dioxygenases (Potrawfke *et al.*, 2001). Moreover, in contrast to RW1, most organisms harboring an angular dioxygenase and capable to mineralize DBF do not grow on DD (Iida *et al.*, 2002a; Kasuga *et al.*, 2001) Based on experiments performed on RW1 and discussed above, it can be speculated that this failure to grow is due to the absence of an effective activity capable to cleave intermediate THBE. Actually, in studies analyzing the transformation of chlorinated dioxins by DBF63 or CA10 only small amounts of substrate were transformed into the respective catechol (Habe *et al.*, 2001a; Habe *et al.*, 2002a; Widada *et al.*, 2002), indicating the accumulation of other intermediates and pathway bottlenecks upstream of catechol formation.

In contrast to the immense information available for transformation of PCB congeners by biphenyl dioxygenases, information on the substrate range of angular dioxygenases regarding the transformation of chlorinated DBFs and chlorinated DDs is still scarce. As expected for Rieske non-heme iron oxygenases, all strains harboring angular dioxygenases and tested in this aspect so far share the capability to transform chlorosubstituted substrate analogues, and generally transformation rate decreased with increasing chloride substitution (Sander *et al.*, 1991; Schreiner *et al.*, 1997). However, the available data do not allow to deduce if certain dioxygenases are characterized by special broad substrate specificity, as the case for example for biphenyl 2,3-dioxygenase from *Burkholderia xenovorans* LB400, which can transform an exceptional broad range of PCB congeners (Mondello *et al.*, 1997).

All strains harboring angular dioxygenases and analyzed in this aspect so far, share the capability to transform 2-chlorodibenzofuran and 2-chlorodibenzo-*p*-dioxin (Fukuda *et al.*, 2002; Habe *et al.*, 2001a; Wilkes *et al.*, 1996), however, differences were observed both in regioselectivity of attack and substrate specificity. As an example, *Terrabacter* sp. strain DBF63, and *Pseudomonas* sp. strain CA10 catalyze exclusively a 5a,6-dioxygenation (Habe *et al.*, 2001b), whereas the RW1 enzyme catalyze both 4,4a- and 5a,6-dioxygenation (Wilkes *et al.*, 1996). 2,7-dichlorodibenzodioxin is not transformed by the *Terrabacter* sp. strain DBF63 enzyme (Habe *et al.*, 2001b) and only with negligible rate by the strain RW1 enzyme (Wilkes *et al.*,1996), whereas it is a good substrate for the *Pseudomonas* sp. strain CA10 (Habe *et al.*, 2001a).

Sphingomonas sp. strain HH69 was shown to be capable to transform 3-chlorodibenzofuran (Harms *et al.*, 1991) which was subject to dioxygenation both at the 4,4a as well as the 5a,6 positions giving rise, after *meta*-cleavage and hydrolysis to salicylate and 3-chlorosalicylate respectively, as reported also for the RW1 derived angular dioxygenase as well as for *Sphingomonas* sp. RW16 (Habe *et al.*, 2001a; Harms *et al.*,

1991; Wilkes *et al.*, 1996; Wittich *et al.*, 1999). However, the transformation 3CDBF by angular dioxygenases of Gram-positive organisms has so far not been described.

1.10 Aims of the work

As described above, oxygenases are key enzymes in the degradation of biarylethers, a substance class that comprises DDs, and DBFs. These enzymes catalyze the initial activation, which, for mineralization to occur, should be a specific angular dioxygenation. Thus far, only a few enzymes are known to catalyze this reaction, and those enzymes have a limited substrate range. Ring-cleavage of intermediate hydroxylated products specifically of THBE as an intermediate in DD degradation is a second specific degradation problem, as extradiol dioxygenases seem to be rapidly inactivated during THBE-turnover.
The present study is thus divided into two sections:

The first aim was to isolate and characterize new strains capable to degrade biarylethers, and to evaluate the capability to transform chlorinated derivatives. Angular dioxygenases of a new isolate were characterized in detail and their substrate specificities/regioselectivities were aimed to be characterized after heterologous expression.

The second aim was a detailed analysis of strain RW1 with regard to extradiol dioxygenases and analysis of their kinetic properties. On the one hand, already sequenced extradiol dioxygenases were characterized for their capabilities to transform THBE. Available mutants, in which DbfB extradiol dioxygenase was inactivated, were characterized and it was aimed to identify the enzyme supporting DbfB in the degradation of DD by RW1.

2 MATERIALS AND METHODS

2.1 Instruments

Agitator	*SM25 Edmund Bühler*
Autoclave	*Tecnomara*
Balances	*PM460 and AE260, Mettler*
Bench centrifuges	*Biofuges Heraeus Sepathec* *Eppendorf centrifuge 5415*
Cell incubators	*Heraeus B5060,EK002*
Centrifuges	*Sorvall Instruments RC5, RC3*
Computers	*Macintosh and PC*
Computer programs	*Microsoft Word, Deneba Canvas, CSC ChemDrawPro, KaleidaGraph, BioEdit, Xnview, Seqassem, EndNote, Gene Codes*
Drying machine	*Heraeus 5042*
Electrophoresis chambers	*BRL Horizon 58*
FPLC	*Controller LCC-500, pump P500, fraction collector 100, (Pharmacia)*
MilliQ water purifier	*Millipore Milli-Q system*
PCR thermocycler	*Perkin Elmer (GeneAmp PCR system 2400)*
PH-meter	*Schott CG804*
Powersupply	*Bachofer*
Protein gels chamber	*Bio-Rad Mini Protein II Cell*
Spectrophotometer	*Beckmann DU-70, Shimadzu UV-2100, Hitachi U 2000*
Ultracentrifuge	*Sorvall Instruments Combi*
Vacum centrifuge	*Savant*
Vortex	*Speed Vac A110 Janke&Kunkel IKA VF2*
Water bath	*Julabo 5B*

2.2 Chemicals and reagents

Most chemicals used in this study were obtained from Sigma and Aldrich, and were of the highest grade available. 2,3-Dihydroxybiphenyl for enzymatic tests was obtained from Wako Chemicals GmbH. 2,2',3-Trihydroxybiphenyl ether and 2,2',3-Trihydroxybiphenyl were available from previous syntheses. 3-Chlorodibenzofuran was kindly provided by Stefan Schmidt. 2-chlorodibenzofuran was obtained from the Sigma-Aldrich library of rare chemicals, and dibenzo-*p*-dioxin from Promochem GmbH. Oligonucleotide primers were obtained desalted from Invitrogen and used without further purification.

Restriction enzymes and reagents for genetic procedures were purchased from New England Biolabs, Boehringer Mannheim, Promega, QIAGEN, Q-BIO gene, EPICENTRE® Biotechnologies, United States Biochemicals and Sigma.

2.3 Bacterial strains, plasmids and culture condition

***Sphingomonas wittichii* RW1** was purchased from the German collection of Microorganisms and cell cultures (No. 6014; Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig; Germany).

M10 and M2 are mutants from strain RW1, where DbfB was inactivated by exchange of the wild type *dbfB* gene in the chromosome of RW1 against a *dbfB* gene interrupted at the single NotI restriction site by the kanamycin resistance gene from pUTXylkE (de Lorenzo *et al.*, 1990), through homologous recombination (Happe, B., unpublished).

***Rhodococcus* sp. ATCC 12674** was purchased from the German collection of microorganisms and cell cultures (No. 43287 =ATCC 12674; Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig; Germany).

***E.coli* BL21(DE3)[LysS]** cells carrying pT7-5RW (Happe *et al.*, 1993), pT7-22, or pT7w4, (D'Enza, 2002) carrying *dbfB*, *edo2*, *or edo3* under the control of the T7 RNA promoter, were available from previous work.

Further *E. coli* strains, phages and vectors used in this study are listed in Table 2.1 and 2.2.

Table 2.1. *E coli* strains and phages used in this study.

Strain and phage	Description	Source
E. coli XL1-blue MRF′	Lac (F′ *pro AB lacI^{q}ZΔM15 Tn10 (Tet^r)*	Stratagene
E. coli XLOLR	Lac (F′ *pro AB lacI^{q}ZΔM15 Tn10 (Tet^r) Su (nonsuppressing)λ^r*	Stratagene
Exassist helper Phage	For *in vivo* excision of the pBK-CMV phagemid from ZAP Express vector with *E.coli* XLOLR	Stratagene
E. coli JM109	Competent cells; F′*proAB lac^q ZΔM15*	Promega
E.coli BL21(DE3)[Lys]	T7 Express *lysY* Competent *E. coli*	(Studier & Moffatt, 1986)

Table 2.2. Vectors used in this study.

Vectors	Properties	Source
pGEM®-T Easy Vector systems	Cloning vector	Promega
Zap Express Vector	Lambda vector; prokaryotic and eukaryotic expression, in vivo excision of pBK. CMV vector	Stratagene
PBK-CMV vector	$Neo^{r}Kan^{r}$co1E1origin;lacZ;CMV^{a} promoter	Stratagene
pUC119	Cloning vector $Amp^{r,}$	Takara Shuzo Co
pRSG43	*E*.coli-*Rhodococcus* shuttle vector	Kindly provided by Michael Schlömann
pDFDE	4.4 Kb by *EcoR1* and *Pst1*sites cloned in pUC119	This study
pDFDR	4.4 Kb by *Pst1* and *EcoR1* sites cloned in pRSG43	This study
pDBFA12a	3 Kb by *HindIII* and *EcoR1* sites cloned in pUC119	This study
pDBFA12	3 Kb by *Xba*1 and *EcoR1* sites cloned in pRSG43	This study
pEDO4	EDO4 cloned in pGEM®-T Easy Vector	This study
pEDO5	EDO5 cloned in pGEM®-T Easy Vector	This study
pEDO6	EDO6 cloned in pGEM®-T Easy Vector	This study
pEDO7	EDO7 cloned in pGEM®-T Easy Vector	This study

2.4 Culture media

The following media were used for culturing strains used in this study:

Complex media:

LB-medium
(Luria Bertani medium)

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g

H_2O to 1L, pH 7.0

NZY Medium

NaCl	5 g
$MgSO_4.H_2O$	2 g
Yeast extract	5 g
NZamine (caseine hydrolysate)	10 g

H_2O to 1L, pH 7.5

GYM streptomyces medium

Glucose	4 g
Yeast extract	4 g
Malt extract	10 g
$CaCO_3$	2 g

H_2O to 1L, pH 7.2

SM buffer

NaCl	5.8 g
$MgSO_4.7H_2O$	2 g
1M Tris-HCl, pH 7.5	50 ml
2% gelatine solution	5 ml
H_2O to 1L	

SOC medium

Tryptone	20 g
Yeastextract	5 g
NaCl	0.5 g
KCl	2.5 mM
Glucose	20 mM
H_2O to 1L, pH 7	

Minimal medium

Components for the minimal medium:

Buffer

$Na_2HPO_4 \times 12H_2O$	14 g
KH_2PO_4	2 g
H_2O to 1L, pH 7.4	

100x Salt-solution

$Ca(NO_3)_2 \times 4H_2O$	5 g
$MgSO_4 \times 7H_2O$	20 g
FeIII-ammonium-citrate	1 g (28 % Fe)
Trace elements solution	100 ml
H_2O to 1L	

Trace elements (sterilized by filtration)

HCL(25%)	1.3 ml
$ZnCl_2$	70 mg
$MnCl_2 \times 4H_2O$	100 mg
H_3BO_4	62 mg
$CoCl_2 \times 6H_2O$	190 mg
$CuCl \times 2H_2O$	17 mg
$NiCl_2 \times 6H_2O$	24 mg
$NaMoO_4 \times 2H_2O$	36 mg
H_2O to 1L	

Liquid medium was assembled by supplementing the buffer with 1% (vol/vol) of salt solution.

Carbon sources were added in appropriate concentrations, usually 2 mM.

Solid mineral medium was assembled by sterilization of 15 g Agar in 900 ml H_2O. This medium was supplemented with 100 ml 10-fold concentrated buffer, 10 ml of salt solution and carbon sources in appropriate concentrations.

Solid LB medium was prepared by addition of 15 g/l of agar to LB medium. Solid GYM streptomyces medium was prepared by addition of 2 g/l $CaCO_3$ and 15 g/l of agar to GYM streptomyces medium. NZY Top Agar was prepared by supplementing NZY broth with 0.7% agarose.

For screening *E. coli* clones carrying vectors with inserts, LB medium or GYM streptomyces medium was supplemented with 15 g/l of agar, with addition ampicillin or kanamycin to a final concentration of 100 µg/ml and 0.5 mM IPTG and 80 µg/ml X-Gal.

2.5 Isolation and identification of DBF degrading bacteria

Soil samples were collected from soil in Kafr El Ziat Egypt around some chemical, insecticides, and pesticides producing factories. 1 g of soil was incubated in 1 L Erlenmeyer flasks containing 100 ml of mineral medium with DBF (2 mM) as the sole source of carbon and energy. After one month of cultivation at 30°C, 10 % of the culture was transferred to fresh medium and cultured for one more month. Dilutions were spread onto minimal medium agar plates supplemented with crystals of DBF in the lid of the plate as sole carbon source. After 7 days of incubation colonies were sprayed with filter sterilized 2,3-dihydroxybiphenyl (DHB) (10 mM). Colonies turning yellow due to extradiol cleavage of DHB were purified by repeated subculturing and streaking on minimal medium agar plates with DBF as sole carbon source.

2.6 Growth of *Rhodococcus* sp. strain HA01 on biarylethers

Cells were usually cultured in Erlenmeyer flasks filled up with no more than 20% of their total volume on a rotary shaker operated at 150 rpm. *E. coli* cells were cultured in LB medium at 30 - 37 °C in liquid medium containing the appropriate selection markers and *Rhodococcus* sp. ATCC 12674 in GYM streptomyces medium at 30°C. *Sphingomonas wittichii* RW1, its mutants M10 and M2, or *Rhodococcus* sp. strain HA01 were cultured at 30°C in LB medium or mineral medium supplemented with the appropriate carbon sources usually at concentrations of 2 mM. Hydrophobic substrates (DBF, DD, their chlorinated derivatives as well as carbazole) were added from 100 mM filter sterilized stock solutions in DMSO and incubation was performed in screw cap sealed Erlenmeyer flasks to avoid evaporation. For growth on hydrophobic substrates on agar plates, the substrates were applied as crystals in the lid of the plates. Growth was usually monitored by following the optical density OD_{600nm}.

To quantify growth rate and substrate disappearance, *Rhodococcus* sp. strain HA01 was grown as described above and cultures harvested during late exponential growth phase by centrifugation at 7000 rpm for 10 min. Cells were washed twice with 50 mM phosphate buffer (pH 7) and resuspended in liquid mineral medium to give an OD_{600nm} of 0.1. Degradation of DBF was tested in sterilized glass tubes containing 2 ml cell suspension (OD_{600nm} = 0.1) and 2 mM of DBF as sole carbon source. The test tubes

were incubated at 150 rpm and 30°C. At each time point, 2 test tubes were analyzed. For the estimation of the colony forming units (CFU) aliquots were serially diluted, 100µl aliquots were plated on solid LB medium and the CFUs counted after 2 days incubation at 30°C. To measure substrate depletion, 0.8 ml from each tube were supplemented with 7.2 ml methanol for extraction of residual DBF from cell walls and to achieve complete dissolution. In control experiments it could be shown that by this method more than 95 % of residual substrate could be extracted. Samples were centrifuged in the test tubes at 9,000 g for 20 min and the supernatant either directly analyzed by reverse phase high-performance liquid chromatography (HPLC) or stored at 20°C until analysis. Uninoculated tubes and tubes without substrate (with DMSO only) served as controls.

2.7 Screening of organisms expressing 2,3-dihydroxybiphenyl 1,2-dioxygenase

2,3-Dihydroxybiphenyl has been shown to be a substrate for many extradiol dioxygenases, including DbfB, Edo2 and Edo3 of *Sphingomonas wittichii* RW1 (D'Enza, 2002; Happe *et al.*, 1993). The *meta*-cleavage reaction product is yellow colored providing an easy colorimetric test for a rapid screening of bacterial colonies carrying 2,3-dihydroxybiphenyl 1,2-dioxygenase activity. A sterile solution 10 mM of 2,3-dihydroxybiphenyl (dissolved in 1 % of the final volume of ethanol, to which water was added to give the final molarity) was sprayed on the bacterial colonies. Positive clones could be easily identified due to yellow coloration around the colonies.

2.8 Biochemical studies

2.8.1 Preparation of resting cells

For preparation of resting cells, *Rhodococcus* sp. strain HA01 was grown in minimal medium with DBF (2 mM) or fructose (2 mM) as sole carbon source at 30°C on a rotary shaker operated at 150 rpm. During late exponential growth, cells were harvested by centrifugation, washed twice with 50 mM phosphate buffer (pH 7.4) and resuspended to an OD_{600nm} of 5 -20.

Similarly, resting cells of *Sphingomonas wittichii* RW1, M2, M10 were prepared after growth in minimal medium supplemented with acetate, salicylate, DBF, or DD as the sole carbon sources (2mM). The cells were harvested during late exponential growth, washed twice with 50 mM sodium phosphate buffer (pH 7.4), and resuspended in 5 ml of the same buffer to give an OD_{600nm} of 3.0.

Rhodococcus sp. ATCC 12674 (pRSG43) and *Rhodococcus* sp ATCC 12674 (pDFDR) or *Rhodococcus* sp. ATCC 12674 (pDBFA12) were precultured by inoculating individual colonies into 10 ml of GYM streptomyces medium supplemented with kanamycine (100 µg ml^{-1}). After overnight culture at 30°C on a rotary shaker (150 rpm), 500µl of the preculture was inoculated into 200 ml of the same medium in 1L Erlenmeyer flasks incubated as above until an OD_{600nm} of 0.6. For induction, this medium was supplemented with 0.5 mM isopropyl-thio-ß-D-galactopyranoside (IPTG). Induced as well as uninduced cells were further incubated until an OD_{600nm} of approximately 1.0 was reached. The cells were

harvested by centrifugation at 5,000 rpm for 10 min at 4°C, washed twice with 50 mM phosphate buffer (pH 7.4) and resuspended in the same buffer to give an OD_{600nm} of 10 - 20.

E. coli JM109 (pRSG43), *E .coli* JM109 (pDFDE), *E .coli* JM109 (pDBFA12a) and *E. coli* JM109 (pDBF12) were precultured by inoculating individual colonies into 10 ml of LB medium supplemented with kanamycine (100 µg ml^{-1}). After overnight culture at 30°C on a rotary shaker (150 rpm), 500µl of the preculture was inoculated into 200 ml of the same medium in 1L Erlenmeyer flasks. Further incubation, induction, and cell harvesting was performed as described above for *Rhodococcus* sp. ATCC 12674 cells.

2.8.1.1 Transformation of DBF, DD, 2-chlorodibenzofuran (2CDBF), 3-chlorodibenzofuran (3CDBF) and carbazole by resting cells

Resting cells of *Rhodococcus* sp. strain HA01, *Rhodococcus* sp. ATCC 12674 (pRSG43) *Rhodococcus* sp. ATCC 12674 (pDFDR), *Rhodococcus* sp. ATCC 12674 (pDBFA12), *E.coli* JM109 (pRSG43), *E.coli* JM109 (pDFDE), *E.coli* JM109 (pDBFA12a) or *E.coli* JM109 (pDBF12) were incubated with DBF, DD, 2CDBF, 3CDBF or carbazole supplied at concentrations of 0.5 – 2 mM in aliquots of 1 ml in 10 ml reagent tubes closed with teflon coated screw caps and incubated at 30°C on an overhead shaker. After appropriate time intervals, one whole tube was analyzed for the amount of substrate remaining. For doing this, 8 ml of methanol were added for extraction of residual substrate from the cell walls and to achieve complete dissolution. Samples were centrifuged in the test tubes at 5,000 rpm for 20 min and the supernatant either directly analyzed by HPLC or LC/MS or stored at -20°C. For quantification of transformation products, 5 ml of cell suspension were incubated in 10 ml reagent tubes as described above. At appropriate time intervals aliquots of 0.5 ml were removed, centrifuged as above, and the supernatant either directly analyzed by HPLC or stored at -20°C.

To inactivate extradiol dioxygenases and thus to accumulate and identify ring-cleavage intermediates during transformation of biarylethers, resting cells of *Rhodococcus* sp. strain HA01 were incubated with the respective substrates as described above, however, in the presence of a well-known inhibitor of these enzymes, 3-chlorocatechol (0.1 mM concentration) (Wittich *et al.*, 1992; Yamazoe *et al.*, 2004b).

2.8.1.2 Transformation of catechol, DHB, and THB by *Sphingomonas wittichii* RW1 and its mutants M2 and M10

Resting cells of *Sphingomonas wittichii* RW1, M2, or M10 were incubated at 30°C with 100 µM of catechol, DHB, or THBE in 2 ml eppendorf tubes containing with 1.5 ml cell suspension. For quantification of substrate depletion, samples (200 µl) were taken at appropriate time intervals, centrifuged, and the supernatant either directly analyzed by HPLC or stored at –20°C.

2.8.2 Preparation of cell extracts

Cell extracts were prepared at 4°C. For determination of activities in *Sphingomonas wittichii* RW1 as well as M2 and M10, 100 ml of cells were harvested during the late exponential growth phase by centrifugation (10,000 rpm, 10 minutes). *E.coli* BL21(DE3)[LysS] (pT7-5RW), *E.coli* BL21(DE3)[LysS] (pT7-22) and *E.coli* BL21(DE3)[LysS] (pT7W4) were precultured at 30°C in LB medium containing 50 mg/ml of ampicillin and 30 mg/ml chloramphenicol and *E.coli* JM109 (pEd04) in LB medium containing 50 mg/ml of ampicillin. 200 ml of the respective media containing additionally 0.5 mM IPTG were inoculated with 0.5-2.0 % of the preculture, incubated as above until an OD_{600nm} of 2.0 was reached and harvested by centrifugation (10,000 rpm, 10 minutes).

Harvested cells were washed twice in 50 mM phosphate buffer (pH 7.4), resuspended in 1 ml of the same buffer and disrupted two times in a French pressure cell (Aminco corp.) operated at 20,000 Ib/in^2 . Cell debris was removed by centrifugation at 30,000 rpm for 30 min. For preparations under limiting oxygen concentrations, the cells and buffers were purged under a stream of N_2.

To test the effect of different metals on extradiol dioxygenase activity, 1 mM $FeSO_4$, $CuCl_2$, $MnCl_2$ or $MgCl_2$ were added from solutions previously purged under a stream of N_2 (Boldt *et al.*, 1995; Boldt *et al.*, 1997; Vetting *et al.*, 2004; Wolgel *et al.*, 1993) before preparation of cell extract.

2.8.3 Determination of protein content

Protein concentrations were determined by the method of Bradford (Bradford, 1976), using bovine serum albumin (BSA) as a standard. The protein preparation was diluted with H_2O to a final volume of 800 µl and mixed with 200µl Bradford reagent (BioRad, Richmond, USA). After 8 minutes incubation at room temperature, the absorbance at λ=595 nm was measured by mean of a spectrophotometer and using zeroing against 800µl H_2O +200 µl Bradford reagent. The obtained absorbance was compared with that of a series of standard BSA dilution solutions of known concentration and the protein content deduced.

2.8.4 Enzyme assays

2,3-Dihydroxybiphenyl 1,2-dioxygenase activity was measured spectrophotometrically by monitoring the formation of reaction products in a total final volume of 1 ml in 50 mM potassium phosphate buffer (pH 8). If not otherwise stated, activities were determined at substrate concentrations of 100 µM. To evaluate the effect of divalent ions (Fe^{2+}, Mn^{2+}, Cu^{2+} or Mg^{2+}) $FeSo_4$, $CuCl_2$, $MnCl_2$ or $MgCl_2$ (1 mM) were added. Specific activities (U/g protein) are expressed as µmol of product formed per minute per gram protein at 25°C.

2.8.5 Analysis of kinetic data

V_{max}, k_{cat} and apparent K_m values of extradiol dioxygenases were determined using substrate concentrations of 0.2 - 5 times the determined K_m values in air-saturated buffer and kinetic data were

calculated from the initial velocities using the Michaelis-Menten equation by non-linear regression (KaleidaGraph, Synergy Software) (Junca *et al.*, 2004).

Turnover numbers (k_{cat} values) were determined using partially purified proteins. To be capable to calculate turnover numbers, the relative amount of extradiol dioxygenase protein in partially purified protein fractions was determined after SDS-Page and Ruthenium II tris (bathophenantroline disulfonate) staining. However, extradiol dioxygenase proteins are known to be severely inactivated due to oxidation of active site Fe^{2+} during protein purifications. To take that inactivation into account, it was assumed that the loss of activity was solely due to such inactivation and that the relative amount of active extradiol dioxygenase corresponds to the ratio of total activity applied/total activity recovered. Finally, turnover numbers were calculated per active subunit, assuming subunit molecular masses of 32.3 kDa, (DbfB), 35.2 kDa (Edo2), 32.3 kDa (Edo3) and 33.1 (Edo4), respectively.

Partition ratios for THBE were determined by HPLC analysis in assays in which limiting amounts of extradiol dioxygenase were added to defined amounts of THBE (usually 100 µM). The amount of extradiol dioxygenase added was such that the enzyme was completely inactivated before 50% of the substrate was consumed. The partition ration was calculated by dividing the amount of THBE consumed by the amount of active enzyme added to the assay (Equation 1).

$$\text{Partition ratio} = \frac{\text{No. of substrate molecules consumed}}{\text{No. of enzyme molecules inactivated}} = \frac{k_{cat}}{\sum J} \quad \text{(Equation 1)}$$

The rate constant of inactivation during catalytic turnover in air-saturated buffer, J, was determined from progress curves obtained with DHB or catechol as reporter substrate (applied in concentrations of 50 – 200 µM) and varying concentrations of THBE (1-100 µM) or 3-Chlorocatechol as inactivator. Whereas 3-chlorocatechol was applied at concentration of 10-400 µM, 0.02 - 0.2 µM, 50 – 800 µM, and 10 – 40 µM to inactivate DbfB, Edo2, Edo3, and Edo4 respectively. To determine inhibition of Edo3 by 3-chlorocatechol the transformation rate of DHB (applied at concentrations of 30, 50, 100 or 200 µM was quantified in the presence of 100-800 µM of 3-chlorocatechol.

The rate constant of inactivation at each combination of substrate concentration and inhibitor concentration, J_s, was determined by fitting Equation 2 to the corresponding progress curves using KaleidaGraph (Synergy Software).

$$P_t = P_\infty(1 - e^{J_s t}) + P_i \quad \text{(Equation 2)}$$

In this equation, P_i is the concentration of product recorded at the start of the assay, and $P\infty$ is the concentration of product subsequently generated during the assay. To minimize the effect of substrate depletion on the rate of the reaction, the assays were performed using amounts of enzyme, which were completely inactivated before 20 % of the substrate were consumed.

The rate constant of inactivation J was obtained using Equation 3

$$J_s = \frac{J\,[Inak]}{K_{mInak}\,(1+[Sub]/K_{mSub})+[Inak]} \quad \text{(Equation 3)}$$

In this equation, K_{mInak} and K_{mSub} are the K_m values for the inactivator (THBE or 3-chlorocatechol) and substrate (DHB or catechol), respectively, in air saturated buffer and J_s at each concentration of inactivator and substrate was determined using Equation 2.

Table 2.3. Substrates used for characterization of 2,3-dihydroxybiphenyl 1,2-dioxygenases. The extinction coefficients of the reaction products and wavelengths used for enzyme activity determinations are indicated.

Substrate	ε [mM^{-1} cm^{-1}]	λ_{max} (nm)	Reference
2,3-dihydroxybiphenyl	13,2	434	(Eltis *et al.*, 1993)
catechol	36.0	375	(Heiss *et al.*, 1995)
3-methylcatechol	16,8	382	(Heiss *et al.*, 1995)
2,2',3-trihydroxybiphenyl	21.0	434	(Happe *et al.*, 1993)

2.8.6 Protein purification

In this study four extradiol dioxygenases of *Sphingomonas wittichii* RW1, namely DbfB, Edo2, Edo3 and Edo4 were partially purified from cell extracts of *E.coli* BL21(DE3)[LysS] (pT7-5RW), *E.coli* BL21(DE3)[LysS] (pT7-22), *E.coli* BL21(DE3)[LysS] (pT7W4) or *E.coli* JM109 (pEDO4), respectively. All purifications were performed aerobically using a fast protein liquid chromatography system (FPLC, Amersham Biosciences) placed in the cold room (4°C). All protein elutions were performed in Tris/HCl (50 mM, pH 7.5) and usually, 10 – 30 mg of protein were separated.

2.8.6.1 Anion exchange chromatography

Cell extracts of *E.coli* BL21(DE3)[LysS] (pT7-5RW), *E.coli* BL21(DE3)[LysS] (pT7-22) or *E.coli* BL21(DE3)[LysS] (pT7w4) were directly loaded onto a MonoQ HR 5/5 (Amersham Pharmacia Biotech) column previously equilibrated with Tris/HCl (pH 7.5, 50 mM). Proteins were eluted with a linear gradient of 0 – 500 mM NaCl over 25 ml in Tris/HCl (pH 7.5 50 Mm) at a flow rate of 0.3 ml/min. Fractions of 0.5 ml were collected and assayed for 2,3-dihydroxybiphenyl 1,2-dioxygenase activity. Active fractions were analyzed by SDS-PAGE as described in (2.8.7).

2.8.6.2 Hydrophobic interaction chromatography

Cell extracts of *E.coli* JM109 (pEDO4), were mixed with 4 M $(NH_4)_2SO_4$ to give a final concentration of 1 M $(NH_4)_2SO_4$ and applied to a SOURCE 15PHE PE 4.6/100 (hydrophobic interaction) column (Amersham Pharmacia Biotech). Proteins were eluted by a linear gradient of $(NH_4)_2SO_4$ (1 M – 0 M) over 25 ml with a flow of 0.25 ml/min. Fractions of 0.5 ml were collected and assayed for 2,3-dihydroxybiphenyl 1,2-dioxygenase activity. Active fractions were analyzed by SDS-PAGE as described in (2.8.7).

2.8.7 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS polyacrylamide gels were used for determining the molecular weight of denaturated protein subunits and to check the efficiency of the protein purifications and were prepared as follows:

Separating gel

Acrylamide concentration	12,5%
Acrylamid/Bis (30%)	4,2 ml
4X lower Buffer pH 8.8 (182 g Tris base, 40 ml SDS (10%9 and 14 ml HCl (conc.) per liter)	2,54 ml
H_2O	2,34 ml
Glycerin	0,86 ml
TEMED	22 µl
APS 10 %	16 µl

Stacking gel

Acrylamide concentration	4%
Acrylamid/Bis (30 %)	0,533 ml
4X Upper Buffer pH 6.8 (61 g Tris Base, 40 ml SDS (10%) and 30 ml HCl (conc.) per liter)	1.0 ml
H_2O	2,467 ml
TEMED	10 µl
APS 10%	16 µl

The protein content of the samples to be analyzed was adjusted to 1 to 30 µg. All samples were diluted 1:1 with the double concentrated loading buffer consisting of 50 µl ß-Mercaptoethanol and 450 µl stock sample buffer (2.4 ml 0,5 M Tris/HCl pH 6,8, 2.0 ml glycerol, 4.0 ml 10% (w/v) SDS and 1 ml 0,1 % (w/v) Bromophenol blue) and denaturated at 95 °C for 7 minutes. PageRuler™ Unstained Protein Ladder (Fermentas) was loaded next to the protein samples for evaluating the molecular weight of the denaturated protein subunits Electrophoresis was performed at 15 mA for the first 10 min and at 25 mA for the rest of the run.

2.8.8 Coomassie brilliant blue staining

Gels were stained in 1% (w/v) Coomassie brilliant blue G-250 in methanol : acetic acid : water (5:1:4) as staining solution (Sambrook *et al.*, 1989) and destained initially in an aqueous solution containing 30% MeOH + 10% acetic acid and followed by destaining in an aqueous solution containing 7% Ethanol + 10% acetic acid.

2.8.9 Ruthenium II tris (bathophenantroline disulfonate) staining

For quantification of extradiol dioxygenase proteins, in partially purified fractions, 0.3 – 15 µg of protein were separated by SDS-PAGE. The gels were fixed in 250 ml of fixation solution comprising 40% ethanol and 10% acetic acid for 30 min. After washing twice for 30 min with 20 % ethanol, the gels were stained overnight in 200 ml of 0·4 µM ruthenium II tris bathophenanthroline disulfonate (Junca *et al.*, 2004;

Lamanda *et al.*, 2004; Rabilloud *et al.*, 2001). Gels were washed in 20 % ethanol 10 % acetic acid to eliminate residual matrix background staining and scanned using a Fujifilm LAS-1000 CCD camera. The fluorescence intensity was integrated, and the relative intensities of the bands corresponding to the *dbfB*, *edo2*, *edo3* and *edo4* proteins were determined using the AIDA software package (Raytest Isotopenmessgeräte GmbH) (Junca *et al.*, 2004; Lamanda *et al.*, 2004).

2.8.10 N-terminal amino acid sequencing

To verify the identity of extradiol dioxygenase proteins in partially purified fractions, proteins were separated by SDS-PAGE and electroblotted onto a PVDF membrane (Millipore) which was stained with Coomassie brilliant blue R250 (0.1 % in a methanol (40 %, vol/vol), acetic acid (10 %, vol/ vol), H_2O (50% vol/vol) solution for 15-30 minutes. The PVDF membrane was destained in the solution without Coomassie and dried over night. The protein bands were excised and N-terminal amino acid sequencing was performed with an Applied Biosystem model 494A Procise HT sequencer.

2.9 Analytical methods

2.9.1 HPLC-analysis

Metabolites were analyzed by injection of 10 µl samples into a Shimadzu (Kyoto, Japan) HPLC system (LC-10 AD liquid chromatograph, DGU-3A degasser, SPD-M10A diode array detector and FCV-10 AL solvent mixer) equipped with a Lichrospher SC 100 RP8 reversed phase column (125 by 4.6 mm, Bischoff, Leonberg, Germany). Methanol-H_2O containing 0.1% (vol/vol) H_3PO_4 was used as eluent at a flow rate of 1 ml/min. The column effluent was monitored simultaneously at wavelengths between 200 and 300 nm by the diode array detector. Methanol-H_2O (72:28) was used for quantification of the depletion of the hydrophobic substrates dibenzofuran, 2-chlorodibenzofuran, 3-chlorodibenzofuran, dibenzo-*p*-dioxin and carbazole, which exhibited typical net retention volumes of 4.3 ml, 5.4 ml, 7.6 ml, and 6.9 ml, respectively.

Methanol-H_2O (60:40) was used for quantification of depletion or accumulation of 2,2',3-trihydroxybiphenyl, 2,2',3-trihydroxybiphenyl ether, 5-chlorosalicylate and 4-chlorosalicylate, which exhibited typical net retention volumes of 1.4 ml, 1.5 ml, 7.4 ml, and 7.6 ml, respectively. Methanol-H_2O (45:55) was used for quantification of depletion or accumulation of salicylate, catechol, and benzoate (net retention volumes of 4.9 ml, 1 ml, and 3.6 ml, respectively).

Transformation products were, if possible, identified by comparison of the absorption spectra and retention volumes with authentic standards. Their concentration was analyzed based on the comparison of the peak area with that of authentic standards of known concentrations.

2.9.2 Characterization of metabolites by HPLC/MS

HPLC-mass spectrometry (MS) was performed using an Agilent 1000 LC system (Agilent Technologies, Palo Alto, CA) equipped with a Nucleosil 120-5-C18 column (125 mm by 2 mm), coupled to a Sciex

API2000 mass spectrometer (Perkin-Elmer Sciex, Foster City, CA) equipped with a TurboIonSpray (ESI) source. Usually 2-10 µL of aqueous solution were analyzed. Elution was performed at a flow rate of 0.3 ml/min using an aqueous solvent system with a linear gradient of 5 mM ammonium acetate (pH 5.5) in 5% acetonitrile to 5 mM ammonium acetate (pH 5.5) in 95 % acetonitrile over 9 ml followed by isocratic elution with 5 mM ammonium acetate (pH 5.5) in 95 % acetonitrile over 3 ml. MS analysis was performed using the Sciex TurboIonSpray source at a temperature of 350 °C in positive and negative ion mode.

2.9.3 *In-situ* ^{1}H-NMR-analysis

For determination the structure of intermediates, *in-situ* ^{1}H-NMR-analysis was used. ^{1}H-NMR spectra could be directly taken from culture supernatants containing the metabolites, as transformations were performed in phosphate buffer thus avoiding interfering signals (Pieper *et al.*, 1993; Pieper *et al.*, 2002). However, in contrast to transformation experiments described above, the substrates were added from a 100 mM stock solution in deuterated DMSO, to avoid interference with DMSO derived signals. The culture supernatant was supplemented with 20% (vol/vol) of D_2O. One-dimensional ^{1}H-NMR spectra were recorded at 300°K on a Bruker AVANCE DMX 600 NMR spectrometer (Bruker, Rheinstetten, Germany) locked to the deuterium resonance of D_2O in the solution. Spectra were recorded using the standard Bruker 1D NOESY suppression sequence with 280 scans each with a 1.8 s acquisition time and 1.3 s relaxation delay. The center of the suppressed water signal was used as internal reference (δ= 4.80ppm).

2.10 Molecular techniques

2.10.1 Genomic DNA extraction

Genomic DNA of *Sphingomonas wittichii* RW1 was isolated from cells pregrown on DBF according to the protocol for bacterial DNA extraction with the FastDNA spin® kit (BIO 101).

Genomic DNA of *Rhodococcus* sp. strain HA01 was extracted from cells pregrown on DBF. 2 ml of the culture was harvested by centrifugation and the pellet suspended in 100 µl sterile H_2O. DNA was then extracted according to the protocol for DNA extraction with the Fast prep DNA Kit for soil (Bio 101). DNA was visualized on 1% agarose gels (2.10.3).

Yield of genomic DNA was determined spectrophotometrically by measuring the absorbance at 260 nm. Purity was determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm (a pure DNA has an A_{260}/A_{280} ratio of 1.7-1.9).

2.10.1.1 Mini preparation of plasmid DNA

Small-scale isolation of plasmid DNA was performed using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. The principle of this kit is the alkaline lysis of bacterial cells followed by adsorption, washing and elution of plasmid DNA on silica membranes.

2.10.2 Polymerase chain reaction (PCR) amplification

2.10.2.1 PCR amplification of 16S rRNA gene

Phylogenetic relationships of the isolates were derived using the nucleotide sequences of the genes coding for 16S rRNA. Nearly complete sequences of 16S rRNA genes (corresponding to positions 15 to 1480 in the *Escherichia coli* numbering system) were determined directly from PCR fragments after purification with the QIAquick PCR purification Kit (Qiagen) by using primers (Table 2.4) and conditions described by Lane (Lane, 1991).

2.10.2.2 PCR amplification of Rieske non-heme iron oxygenase encoding genes

2. 10.2.2.1 Design of primers

Primers DOα-2 and DOα-3 (Iida *et al.*, 2002a) and primers RieskeF and RieskeR (Kasuga *et al.*, 2001) have previously been designed on the basis of conserved amino acid sequence motifs of α-subunits of the terminal oxygenases of Rieske non-heme iron oxygenases.

Further PCR primers (Table 2.4) to amplify gene fragments encoding α-subunits of Rieske non-heme iron oxygenases were designed based on conserved amino acid and nucleic acid sequences found in a collection of sequences retrieved from the GenBank (Benson *et al.*, 1999) and aligned using ClustalX version 1.83, comprising the CDS or protein databases used by (Nam *et al.*, 2001) to categorize, by sequence comparisons, the Rieske non-heme iron oxygenase families. Primers Dxn1F and Dxn2 were designed to detect the α subunit of the dioxin dioxygenase of strain RW1, and were modified with degenerated positions to integrate the codon usage of the relatively scarce amino acid sequences stretches this sequence has in common with its closest relatives *Rhodococcus* sp. strain M5 *bpdC1*, *Rhodococcus erythropolis* TA421 *bphA* and *Rhodococcus globerulus* P6 *bphA*, in order to be capable to detect similar genes in bacterial isolates. Likewise, primers Hamdy1 and Hamdy2 where designed based on conserved amino acid sequences of ferredoxin reductase of strain RW1, *Rhodococcus* sp. strain M5 bpdC1, *Rhodococcus erythropolis* TA421 bphA and *Rhodococcus globerulus* P6 bphA. Primers FPST1yk3, RECOyk3, FHIND3DBF63, RECODBF63, and FXBA1DBF6 were designed with recognition sequences for the restriction endonucleases Pst1, *EcoR1*, *HindIII*, and Xba1, respectively, and served for directional cloning of amplified PCRs product into the pRSG43 shuttle vector (kindly provided by Michael Schlömann) and pUC119 (Kasuga *et al.*, 2001). In Table 2.4., the artificial restrictions sites introduced by the primers are shown underlined and italic. Primers pdfBF1/pdfBR1, pdfBF2/pdfBR2, Edo2F1//Edo2R1, Edo2F2/Edo2R2, Edo3FHH/Edo3RHH, FedoN16RW1/FedoN16RW, 5fedorw1/5Redorw1, fedorw1/6Redorw1, 7fedorw1/7Redorw1 were designed for the specific amplification of gene fragments of the *dbfB, edo2, edo3*, *edo4*, *edo5*, *edo6*, *edo7* genes of strain RW1, using the corresponding CDS as input in the online program Primer3, available at: http://frodo.wi.mit.edu/. All the other primers were designed based on sequence information obtained in this study and used for gene walking to obtain sequence information up- and downstream of the previously obtained fragments.

2. 10.2.2.2 PCR reactions and conditions

PCR reactions were performed with two kinds of polymerases. *Taq* DNA polymerase (Promega) was used for amplification of DNA fragments < 4 kb in length, whereas *Pfu* DNA polymerase (MBI Fermentas)

Table 2.4. Primers used in this study.

Primers	Sequence
16F27	5′-AGAGTTTGATCMTGGCTCAG-3′
16F530	5′-TTCGTGCCAGCAGCCGCGG-3′
16R518	5′-CGTATTACCGCGGCTGCTGG-3′
16F945	5′-GGGCCCGCACAAGCGGTGG-3′
16R1807	5′-CTCGTTGCGGGACTTAACCC-3′
16R1492	5′-TACGGYTACCTTGTTACGACTT-3′
Rieske F	5′-TGYCGBCAYCGBGGBGGSAWG-3′
Rieske R	5′-CCAGCCGTGRTARSTGCA-3′
DO-α2	5′-TGYHSNTAYCAYGGNTGG-3′
DO-α3	5′-TCNRCNGCNARYTTCCARTT-3′
M13F	5′-TCCCAGTCACGACGTCGT-3′
M13R	5′-GGAAACAGCTATGACCATG-3′
YdbfDO-F1	5′-AGGTCTGCCGCGCCGACTGG -3′
YdbfDO-F2	5′-GAACGCGAAGGCCTTCAC-3′
Hamdy1	5′-GAYAGMGGBGGKCGYTSRTASGG-3′
Hamdy2	5′-GCSGCVRCYTCSRRNCCG-3′
Alpha FY1	5′-CATCAACTTGGTGCCAGCGCAC-3′
Alpha RS1	5′-TTGTAGTAGGCGTATTCCTCGC-3′
Alpha F2 S	5′-TCCACGACAGCTCGGTCCTGC-3′
FerredoxinR1S	5′-CCGGTGCGCAAGTTGAACTTA-3′
AlphaF546	5′-GGGGGGATATTTGGCCTCACCC-3′
AlphaR1008	5′-GCCTTGGCCAGCTGCTTTCA-3′
AlphaF1500	5′-ATCGGTGCTGCTGTCTGAGTTC3-3′
FerredoxinR2000	5′-GTGTCCCACTCCCATCTCGAC-3′
FerredoxinredF3000	5′-ATCGACGATACGTGCACCCAC-3′
FerredoxinredR3500	5′-CGGTGTGATGGCCGGTGGGGGG-3′
FerredoxinredF4023	5′-GCCGGTGCCGCTGGCCGGCGT-3′
FerredoxinredR4600	5′-GGTCACGCCAGGTCGGACGAC-3′
FerredoxinredF4700	5′-ATCGAGGGACGAGTTCCCGTT-3′
FerredoxinredR5211	5′-CTGCAGTCCAAGCCGCCGACG-3′
F3450	5′-GGACAGCCCGGGGCGGCGGTGT-3′
R3750	5′-TAGAGGATCCGACGCGGCGCG-3′
R4900	5′-TGCGGGTGGACCGGGCGACG-3′
Dxn1 F	5′-TGYASNTAYCAYGGVTGG-3′
Dxn2 R	5′-TBVGGNCCVYKNGGVTGCC-3′
F1896	5′-GGAACTACGACCACGGCTAC-3′
Dbf F2170	5′-CCCTCCCATCTCACCTCAC-3′
Dbf R3600	5′-GACATCCTCGGTGAACAGGT-3′
Dbf F3200	5′-GGATCAAGTTCGGCGTCTT-3′
Dbf R4600	5′-GTACTCGATCTGCGGCTTGT-3′
Dbf F 4440	5′-GCCAGGCGAGCTCTTACC-3′

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Dbf R5900	5′-GGAGTTGATCTGGCCCATCT-3′
R6880ed01.2	5′-CAACATCGGCGGTAGCAC-3′
FPST1yk3	5′-CACGACGA*CTGCAG*CGGTGTGAT-3′
RECOyk3	5′-CTACTCTTC*GAATTC*CTGCGGCATG-3′
FHIND3DBF63	5′-TGACAGCG*AAGCTT*CAGTGATACC-3′
RECODBF63	5′-GTACCGG*GAATTC*TCCACCAGT-3′
FXBA1DBF63	5′-TGACAGCG*TCTAGA*CAGTGATACC- 3′
FYK3RNA	5′-CAACGTGTTCCCCAACTTCT-3′
RYK3RNA	5′-GGTCGATTACCTCGGTCGTA-3′
F DBF RNA	5′-TCTACCGCAAGGAATTGGAC-3′
R DBF RNA	5′-ATCCCGACGTCGTTCTGATA-3′
PdfBF1	5′TGATGTTCTGGAGCAAATGG 3
PdfBF2	5′ATAGCGGAACAGGTGGAGAA3′
PdfBR1	5′ CACCAAAGCCCAACTCAACT 3′
PdfBR2	5′ATCCAACATGCCAACTTTCC 3′
Edo2F1	5′ CCTGGGCTATCTGGGTTTCT 3′
Edo2F2	5′CCTGGGCTATCTGGGTTTCT3′
Edo2R1	5′ GTCTCCTTCAGCTGCCATGT 3′
Edo2R2	5′ATGTGGGTGAAATGCACCTT 3′
Edo3FHH	5′CGGCTATCTCAGCTTCACCT 3′
Edo3RHH	5′CGGTAGAAGTCGTGGGTCTC 3′
FedoN16RW1	5′ GTTTCGATCCATGGTGACG 3′
RedoN16RW1	5′ CGCCATTTTCTGAATGCAC 3′
5fedorw1	5′ GCGTACTGATTTGCCGATTT 3′
5Redorw1	5′ TTGTCCAATGTCGTCACGTT 3′
6fedorw1	5′ GCCTATGGCGAGTTCCTGT 3′
6Redorw1	5′ CATGGCGCTCTCCTATCC 3′
7fedorw1	5′ GAACTGCGCAGCCTTTATG 3′
7Redorw1	5′ CCATTTGCCATGTTGTTCAG 3′

was used for the amplification of PCR fragments > 4 kb, or in cases where the introduction of mutations had to be minimized, such as in the cloning of full ORFs for protein expression. For optimization purposes, different concentration of $MgCl_2$ (1.5 – 5 mM) were used in gradient PCR reactions with annealing temperatures of 47,7 °C -59.7 °C). PCR amplifications were usually performed on a AB9700 thermal cycler (Applied Biosystems) in a final volume of 50 µl, containing 0.5 µM of each primer (Invitrogen), 0.2 mM of an equimolar dNTPs mix, 1X PCR buffer (Promega) supplemented with 1.5 mM $MgCl_2$, 0.4 µM BSA, 2.5 U of *Taq* DNA polymerase (or additionally 0.5 U of *Pfu* DNA polymerase) and, as template, 1µl of genomic DNA with a concentration between 0.1 to 10 ng. PCR conditions comprised an initial denaturation (95 °C, 5 min) followed by 35 cycles of denaturation (94°C, 1 min), annealing (temperature optimum for the primer set, used 1 min), elongation (72 °C, with elongationa time depending on the expected product size, of 1 min per 1 kb to be polymerized), and a final elongation for 10 min at 72°C.

The PCR products (4 µl of the PCR reaction) were checked for integrity by agarose gel electrophoresis (2% agarose, 1x TAE buffer) and ethidium bromide staining. In case multiple PCR products were

observed, touchdown PCR was applied to increase specificity. Touchdown PCR consisted of an initial denaturation (95 °C for 5 min), followed by 10 cycles of denaturation (95 °C, 45 s), annealing (68 °C, 70 s) and elongation (72 °C, 90 s), followed by 25 cycles with an annealing temperature of 58 °C (60 s each) and a final elongation step (72 °C for 5 min).

2.10.3 Gel electrophoresis

DNA fragments were separated and their sizes determined by electrophoresis on 1-2% agarose gels. The necessary amount of agarose was dissolved in 1x TAE buffer, melted and poured into an electrophoresis chamber, A volume of 10% (v/v) loading buffer (60% glycerin, 0.3 % xylolcyanol 0,3 % bromophenolblue) was added to the DNA samples. Samples were loaded onto the gel and the electrophoresis was performed at 20-100 Volt/cm in 1x TAE buffer. DNA was visualized by soaking in a dilute solution of ethidium bromide.

2.10.4 DNA extraction from agarose gels

After separation on agarose gels, DNA fragments of interest were excised from the gel by mean of a lancet, weighted and treated by mean of the Qiagen II Agarose Gel Extraction Kit. All buffers were provided by Qiagen GmbH (Hilden, Germany). The extraction was performed following the protocol suggested by the provider.

2.10.5 DNA sequencing and homology research

PCR products were purified with a QIAquick PCR Purification Kit and sequenced using an ABI PRISM BigDye Terminator v1.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Primers used for sequencing reactions were the same as those used in the original PCR, except in case of inserts in the pGEM®-T Easy Vector (Promega), which were amplified and sequenced with vector-specific M13–forward and M13-reverse primers (Sambrook *et al.*, 1989). Raw sequence chromatograms from both strands were assembled with Sequencher software version 4.0.5 (Gene Codes Corporation). DNA and protein similarity searches were performed using BlastN and BlastP programs from the National Center for Biotechnology Information website. 16s rRNA gene nucleotide sequences were aligned using ClustalW implemented in MEGA software version 3.1 (Kumar *et al.*, 2004). Nucleotide sequences of aromatic dioxygenases were translated and aligned with the functions available in the same software package. Phylogenetic trees were constructed using the Neighbor-Joining (N-J) algorithm. Distances were generated using the Kimura Matrix, and tree stability was supported through Bootstrap analysis (100 replicates). N-J trees were visualized with NJplot program available at http://pbil.univ-lyon1.fr/software/njplot.html. and TreeView version 1.6.6 available at http://taxonomy.zoology.gla.ac.uk/rod/treeview.html. Vectorial representations were imported and edited on Microsoft Office Powerpoint program.

2.10.6 Enzymatic restriction of DNA

Restriction enzymes and reagents for genetic procedures were purchased from New England Biolabs. This procedure was intended when directional cloning on custom non commercial vectors was desired (pRSG43 and pUC119) (Kasuga *et al.*, 2001).

In the first reaction, 1µg DNA (PCR Product or Vector) dissolved in 10 - 20 µl 1x NEB buffer (*EcoR1*) solution was digested with 1 Unit *EcoR1* restriction enzyme for 2 hours at 37°C in the presence of 100 µg of BSA added to the restriction reaction, the enzyme was inactivated by heating at 65 °C for 10 min in a water bath. The resulting DNA fragments were analyzed on an agarose gel (2.8.5). Fragments of interest were excised from the gel, purified (2.8.6), and subject to a 2nd restriction. As described above, purified DNA was dissolved in 20 - 30 µl H_2O –NEB buffer (specific for the applied enzyme) and digested with 0.5-2 Units restriction enzyme over 1-2 hours at the optimal reaction temperature for the enzyme. After inactivation by heating at 65 °C for 10 min in a water bath, DNA fragments were analyzed on an agarose gel (2.10.3). Fragments of interest were excised from the gel, purified (2.10.4), and used for ligation (2.10.7).

2.10.7 Ligation of DNA fragments

Ligation of purified DNA fragments into digested vector was carried out in a total volume of 20 µl, comprising 12 µl digested and purified DNA fragment (0.5 µg/µl) 4 µl vector (0.2µg/µl), 2 µl T4 DNA ligase (5 U/µl, Roche) and 2 µl ligase buffer provided with T4 ligase. The ligation reaction mixture was incubated over night at 4°C. One part of the ligated material was checked for integrity by agarose gel electrophoresis and the other used directly for transformation.

2.10.8 Colony PCR

To identify colonies that contain plasmids with the desired insert of ligated DNA, a well isolated transformant colony was picked from an agar plate, transferred to 100 µl EB buffer, vortexed, boiled for 10 min, centrifuged at 13,0000 rpm for 5 min, and stored on ice for 2 min. 5 µl of the supernatant were used in a 50 µl PCR reaction for amplification of the desired insert.

2.10.9 Transfer of DNA into recipient microorganisms and screening methods

2.10.9.1 Transformation by heat shock

For each transformation, 50 µl of freshly thawed *E*.coli JM109 competent cells (Promega) were mixed with approximately 50 ng DNA (Vector + insert) and kept on ice for at least 30 min before being heat shocked for 45 seconds at 42 °C in a water bath. After the heat shock, 950 µl SOC medium was added to the cell-DNA mixture. After incubation at 37°C for 1.5 hours, cells were plated on LB/ampicillin/IPTG/X-Gal plates (2.4) in case of using pGEM®-T Easy Vector System or on LB/ kanamycin/IPTG/X-Gal plates (2.4) in case of using pRSG43 *E.coli- Rhodococcus* shuttle vector.

2.10.9.2 Preparation of electro-competent cells of *Rhodococcus*

A seed culture of *Rhodococcus* sp. ATCC 12674 was prepared by inoculating one colony of the culture into 10 ml of GYM *Streptomyces* medium (4.2). The seed culture was incubated with shaking at 30°C for 24 hours. A flask containing 50 ml of GYM *Streptomyces* medium was inoculated with 1ml of the overnight grown seed culture and incubated at 30°C with shaking until an OD_{600nm} of 2 - 4 was reached (approximately 24 hours). To ensure that the culture was not contaminated, a loop full of the culture was streaked out on GYM *Streptomyces* medium agar plates and incubated at 30°C. The remaining culture was harvested by centrifugation at 5,000 rpm for 7 min at 4°C and the pellet re-suspended in 30 ml of ice cold 10 % glycerol. The centrifugation procedure was repeated and the cell pellet re-suspended in 15 ml ice cold 10 % glycerol, followed by centrifugation and re-suspension of the pellet in 2.5 ml ice cold 10 % glycerol and centrifugation and re-suspension in 600 µl ice cold 10 % glycerol. Finally, 100 µl aliquots of the electrocompetent *Rhodococcus* cells were frozen at -70 °C for long term storage.

2.10.9.3 Electroporation of *Rhodococcus* cells

The ligation product (2µl) was placed on ice and supplemented with 100 µl of freshly thawed electrocompetent *Rhodococcus* cells in a microfuge tube, followed by gentle mixing without generating any air bubbles. In another microfuge tube, 100 µl of electrocompetent *Rhodococcus* cells only were placed to serve as a negative transformation control. For a second control experiment, 2µl of pRSG43 vector (0.5 µg/µl) and 100µl of electro-competent cells of *Rhodococcus* were mixed as above and used as positive transformation control. All three mixtures were separately transferred into chilled electro-cuvettes (BIO RAD).

Electroporation was carried out using Gene Pulser^{R} II Electroporation System (BIO RAD) at 2500 V, 25 µF, and 200 Ohm. After electroporation, 400 µl of GYM streptomyces medium was added to the cuvettes. The mixture was transferred into falcon tubes and incubated at 30 °C with shaking for 4 to 5 hours. After incubation, 100 µl of the mixtures were plated separately on GYM streptomyces medium agar plates containing 100 µg/ml of kanamycin to select for *Rhodococcus* cells transformed with shuttle vector pRSG43 or with pDFDR or pDBFA12. The negative control was also plated on GYM streptomyces medium agar plates containing 100µg/ml of kanamycin (4.2). The plates were incubated for 4 days at 30°C. Colonies of *Rhodococcus* supposedly containing pDFDR or pDBFA12 were tested for the presence of the proper insert by colony PCR (2.10.8).

2.10.10 Construction of a genomic library from RW1

2.10.10.1 Partial digestion of RW1 genomic DNA with restriction enzymes

A total of 270 µl genomic DNA (0.85 µg/µl) was supplemented with 30 µl 10X restriction buffer. 49 µl of this DNA solution was supplemented with 1µl of *Sau*3A (4 U/µl), mixed thoroughly and placed on ice. A serial dilution was performed by adding 25 µl of the *Sau*3A containing reaction mixture to 25 µl of DNA

solution, followed by mixing and respective further dilution for 8 times. A 10^{th} reaction tube contained DNA solution only.

Tubes were then incubated at 37°C for exactly 1 h, followed by inactivation of the enzyme by heating at 65 °C. 4 µl of the digestion mixtures were subjected to gel electrophoresis on 1% agarose gels at 100 V for 1h to check for the optimal restriction enzyme concentration giving fragments of approximately 5 kb in size.

2.10.10.2 Size fractionation of DNA fragments

Fractionation of the digested DNA was performed by centrifugation at 27,000 rpm and 4°C for 24 h in 5 ml 10 - 40% sucrose gradient tubes. After centrifugation, 24 fractions of 400 µl each were collected in reaction tubes and supplemented with 200 µl each of a 20 % PEG solution in 2.5 M NaCl to precipitate the DNA. After 24 h incubation at room temperature, tubes were centrifuged for 30 min at 12,000 rpm and the pellets washed with 70 % ethanol, followed by centrifugation. Pellets were suspended in 12 µl H_2O and aliquots of 2 µl subjected to electrophoresis in 0.4 % agarose gels at 4°C and 60 V for 5h.The gel was stained with 0.5x TAE/CYPER Gold stain (Invitrogen) for 60 min at 4°C, destained in 0.5x TAE for 20 min, and visualized under UV light.

2.10.10.3 Ligation and packaging the insert

The recovered fraction of 5-10 kbp DNA fragments was ligated into ZAP Express vector (Stratagene) according to the manufacturers protocol. The Gigapack III Gold Packaging Extract (Stratagene) was used for packaging 200 ng of the ligated DNA into functional phage particles. The resulting supernatant containing the phage was kept at 4°C until tittering had been achieved.

2.10.10.4 Tittering the packaging reaction

An *E. coli* VCS257 glycerol stock was streaked on LB agar, incubated overnight at 37 °C and a single colony used to inoculate 3 ml LB medium supplemented with 10 mM $MgSO_4$ and 0.2 % (w/v) maltose. After growth at 37°C to an OD_{600nm} of 1.0, cells were harvested and resuspended in sterile 10 mM $MgSO_4$ to an OD_{600nm} of 0.5. 200 µl aliquots of the *E. coli* VCS257 suspension were supplemented with 1 µl of different dilutions of the phage suspension (serial dilution was performed in SM buffer, and the mixture incubated at 37°C for 15 min). To each aliquot, 4.5 ml of NZY top agar (48°C) were added and plated on 200 mm NZY agar plates, which were incubated overnight at 37°C. The plaques were counted to determine the concentration of phages (pfu/ml) in the library.

2.10.10.5 Phage plating and screening

For each plate, 1 µl of a phage solution containing approximately 5 $x10^4$ pfu was added to 600 µl of the *E. coli* VCS257 host strain followed by incubation at 37°C for 15 min. To each mixture, 8.5 ml of NZY top agar (48°C) were added and plated immediately onto a prewarmed NZY agar plate (22 cm X 22 cm). The plates were allowed to settle for 10 minutes, inverted and incubated at 37°C for about 8 hours.

The plates were then sprayed with 10 mm 2,3 dihydroxybiphenyl (2.7) to screen for plaques containing extradiol dioxygenase activity and thus turning yellow after spraying.

2.10.10.6 Plating and screening of excised phagemids

A fresh culture of E. *coli* XLOLR in 50 ml NZY broth was grown overnight at 30°C. Cells were harvested by centrifugation at 5,000 rpm, 4 °C for 5 min and the pellet resuspended in 10 mM $MgSO_4$ to give an OD_{600nm} of 1. 10 µl and 100 µl, respectively, of the prepared phage stock solution (1.5 $x10^3$ pfu/µl) were added to 2 separate sterile Falcon tubes, each containing 200 µl of *E. coli* XLOLR cells. The tubes were then incubated at 37°C for 15 min. To each tube, 300 µl of NZY broth medium were added and the tubes were further incubated for 45 min. 200 µl of each mixture were plated on LB-kanamycin (50 µg/ml) agar plates and the plates incubated at 37°C overnight. Colonies on the agar plates were supposed to contain the PBK-CMV double stranded vector with the cloned DNA insert. The colonies were sprayed with 10 mM 2,3 dihydroxybiphenyl, for screening as mentioned (2.7) for extradiol dioxygenase activity.

Phagemids were isolated from the positive colonies by using the QIAprep Spin Miniprep Kit (Qiagen) (2.10.1.2). The PCR was performed with primers developed to specifically amplify *dbfB*, *edo2*, or *edo3* genes (2.10.2.2.1).

2.10.11 Preparation and screening of a fosmid library

A genomic library was prepared in pCC1FOS according to the manufacturer´s recommendations (Epicentre). DNA of strain *Sphingomonas wittichii* RW1 was isolated as mentioned above (2.8.1.1). The purified genomic DNA was randomly sheared by pipetting to give approximately 40 kb fragments, end-repaired with a T4 DNA polymerase and T4 polynucleotide kinase enzyme mix according to the protocol by EPICENTRE® Biotechnologies, and subsequently separated by pulsed-field gel electrophoresis (1% low-melting-point agarose, 0.5x TBE, 15 °C, 6 V/cm, 120° angle), with switching times ramps from 0.5 s to 1.5 s over 20h. DNA fragments were excised, purified and ligated into pCC1FOS. Ligated DNA was packaged with MaxPlax Lambda Packaging Extracts to form the fosmid library. Packaged fosmids were transduced into *E. coli* strain EPI300-T1 and spread on LB agar plates containing 12.5 µg/ml chloramphenicol. A total of 3000 individual clones were analyzed by spraying with 10 mM 2,3 dihydroxybiphenyl. 95 Positive colonies were analyzed by colony PCR (2.8.2.2.3) for the presence of known extradiol dioxygenase encoding genes (*dbfB*, *edo2,* and *edo3* genes respectively), and also for the extradiol dioxygenase genes cloned in this study (*edo4, edo5, edo6*, and *edo7*).

Positive individual clones were transferred to 96-well microtiter plates containing 100 µl LB-chloramphenicol broth per well, grown overnight and stored at -70°C.

2.10.11.1 Screening the fosmid library for extradiol dioxygenases activity

For preparation of cell extracts, clones were grown in 5 ml LB-chloramphenicol broth overnight at 37°C. Subsequently, 5 ml of fresh LB-chloramphenicol broth were supplemented 5 µl of CopyControl Induction

solution (Epicentre) and inoculated with 500 ml of the overnight culture. After 5 h of incubation (150 rpm, 37°C), cells were harvested by centrifugation (3000 rpm, 15 min, 4°C), re-suspended in 850 ml 50 mM phosphate buffer (pH 7.5) and disrupted by addition of 150 µl B-PER® Bacterial Protein Extraction Reagent (Pierce). For each sample, cell debris was removed by centrifugation (3000 rpm, 15 min, 4°C) and the supernatant supplemented with DHB (1 mM). Activity was monitored by following formation of ring-cleavage product at 434 nm.

To screen for transformation of THBE, clones were grown separately in 96-well plates with each well containing 1 ml LB-chloramphenicol broth and incubated at 37°C overnight. Subsequently, 200 µl of the overnight cultures were transferred to fresh 96-well plates containing 200 µl of LB-chloramphenicol broth and 0.4 ml CopyControl Induction solution (Epicentre). Cells were grown with vigorous agitation (200 rpm) at 37°C for 5 h. After centrifugation (3000 rpm, 15 min, 4°C), the cells were washed with 0.4 ml 50 mM phosphate buffer (pH 7.5) and resuspended in 0.2 ml of the same buffer supplemented with THBE (50 mM). Plates were incubated overnight at 30°C and cell free supernatants analyzed by HPLC for THBE depletion.

2.10.12 Cloning of PCR products

Putative loci on the RW1 genome producing significant similarity values with extradiol dioxygenase sequences were amplified and the PCR products were applied for direct cloning of PCR products after amplification (non directional TA dependent cloning). Approximately 300 ng of the PCR product purified with Qiaquick PCR purification kit (QIAGEN), were used in ligation reactions using the pGEM-T Easy Vector System (Promega), in order to obtain antibiotic resistant *E. coli* transformants.

2.10.13 RNA Technology

2.10.13.1 RNA isolation

For gene expression studies, *Rhodococcus* sp. strain HA01 was grown on DBF (2 mM) as sole carbon source. To assess constitutive expression, the strain was grown in parallel on fructose (2mM). Cultures were harvested during exponential growth by centrifugation (7000 rpm, 10 min). In case of DBF-grown cells, residual crystals of DBF were removed by filtration (595 1/2 filter paper, Schleicher& Schuell) before harvest. The cell pellets were suspended in 50mM phosphate buffer (pH 7.4) to give an OD_{600nm} = 2.

Total RNA was isolated by a modified version of the method of Siering and Ghiorse (Siering and Ghiorse, 1997). The cell suspension (3 ml, OD_{600nm} = 2) was centrifuged for 15 min at 7000 rpm, the cells suspended in 700 µl of sterile nuclease-free water (Qiagen), transferred to lysing matrix tubes provided with the Fast DNA spin kit for soil (Bio 101 Systems, Q-BIOgene) and centrifuged for 10 min at 13,000 rpm. Supernatants were removed, 750 µl of phosphate buffer (0.12 M, pH 7.0) and 500 µl of acidic phenol (pH 4.6) (Roth) were added and cells were disrupted by beat beating for 45 seconds in a FastPrep FP120A instrument (Bio101 Systems, Q-BIOgene). After centrifugation (5 min, 10,000 rpm, 4°C), the aqueous phase was purified by 500 µl of acidic phenol (pH 4.6) and washed with 500 µl chloroform: isoamylalcohol

(24:1) to remove the remaining phenol. After centrifugation as above, the aqueous phase was supplemented with 10 % of 3 M sodium acetate and two volumes of ethanol and the mixture stored on ice for 30 min, followed by centrifugation (30 min, 13,000 rpm, 4°C). The precipitate was washed with 70% ethanol, centrifuged (5 min, 13,000 rpm, 4°C) and the pellet dried for 3 min in a vacuum centrifuge and resuspended in 30 µl RNase free water. The extracted products were analyzed by electrophoresis in 1.5% agarose gels at 80 V for 50 min. For the preparation of RNA, DNA was digested with RNase-free DNase I (Roche). DNase I (75 unit) was added to 20 µl of above nucleic acid preparation and incubated for 2 h at room temperature, followed by RNA purification using a RNeasy Kit (Qiagen) according to the manufacturer's instructions.

2.10.13.2 cDNA synthesis and RT-PCR

cDNA was synthesized from 1µl of total RNA using a first strand cDNA synthesis Kit for RT-PCR (Roche) according to the manufacturer's instructions. The reverse transcription reaction mixtures were serially diluted (3.2-fold) with nuclease-free water (Qiagen) and 1 µl of each dilution was subjected to amplification by PCR using the primer set FYK3 RNA/RYK3RNA for *dfdA*, and FDBFRNA/RDBFRNA for *dbfA* as described above (2.8.2.2.2). Amplification products were separated in 1% agarose gels and stained with ethidium bromide. Product bands were purified from agarose gels using a QIAquick PCR Purification Kit (Qiagen) and sequenced to verify their identity.

3 RESULTS

3.1 Biochemical and genetic analysis of the dibenzofuran degrader *Rhodococcus* sp. strain HA01

3.1.1 Isolation and characterization of DBF-degrading bacteria

Soil samples were collected from soil in Kafr EL-Ziat (Egypt) in the vicinity of chemical, insecticide, and pesticide manufacturing plants. Enrichment cultures (see 2.5) were set up with 2 mM DBF as sole source of carbon source and energy. After two transfers, aliquots were plated on agar plates with DBF as sole carbon source. Purified single colonies exhibiting yellow coloration after spraying with 2,3-dihydroxybiphenyl were purified by streaking on agar plates with DBF as sole carbon source. One predominant colony morphotype, which grew rapidly at 30°C and exhibited a slightly red color was selected for further analysis and designated strain HA01. Another colony morphotype, which grew moderately at 30°C and exhibited white color, was designated strain HA02.

For characterization of the isolates, the nearly complete 16S rRNA gene sequence of isolate HA01 (corresponding to position 15-1494 according to the *E. coli* numbering system) and a partial 16S rRNA gene sequence of isolate HA02 (corresponding to position 530-1087) were determined (2.10.2.1). Analysis using the RDPII database revealed that the isolate HA01 belongs to the genus *Rhodococcus* whereas isolate HA02 belongs to the genus *Paenibacillus.* Concerning its 16S rRNA gene sequence, HA02 showed highest similarity (>99% sequence identity) with *Paenibacillus* sp. YK20 (accession No AB091337), and was closely related to *Paenibacillus* sp. strain YK5, which was very recently reported as DBF degrader (Iida *et al.*, 2006) and *Paenibacillus naphthalenovorans*, which has previously been reported to be able to grow on naphthalene (Daane *et al.*, 2001; Daane *et al.*, 2002).

The phylogenetic relationships between strain HA01 as well as type strains of the genus *Rhodococcus* and related taxa were determined by a neighbor-joining distance analysis of their 16S rRNA gene sequences (Fig. 4.1). *Rhodococcus* sp. strain HA01 was selected for further studies and analysis, as so far no *Rhodococcus* strains capable to mineralize DBF had been described. Concerning its 16S rRNA gene sequence, strain HA01 showed highest similarity (>98% sequence identity) with the type strains of *Rhodococcus rhodochrous*, and *Rhodococcus pyridinivorans* and was most closely related to *Rhodococcus* sp. strain T104, which has previously been reported to be able to grow on biphenyl as well as on terpenoids including limonene, cymene, pinene, and abietic acid as the sole carbon and energy source (Hernandez *et al.*, 1997).

3.1.2 Growth of *Rhodococcus* sp. strain HA01 on DBF

Rhodococcus sp. strain HA01 can utilize DBF as a sole source of carbon and energy (Fig. 3.1). Growth was accompanied by an increase in colony forming units and concomitant decrease in the concentration of

DBF was verified by HPLC analysis. No growth was observed in the control test tubes containing DMSO only. HA01 grew rapidly at 30°C, with a doubling time of 3 hours.

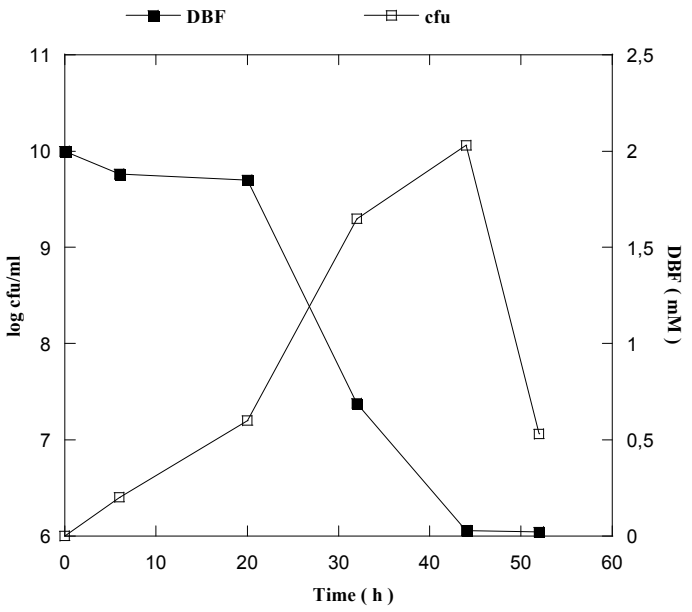

Fig. 3.1. Growth of *Rhodococcus* sp. strain HA01 on 2 mM DBF as a carbon source. Growth was monitored by following colony-forming units (cfu) and substrate depletion was assessed by HPLC.

3.1.3 Transformation of DBF by resting cells of *Rhodococcus* sp. strain HA01

For quantification of the transformation rate of DBF, cells pregrown on DBF were resuspended to an OD_{600nm} = 5, incubated with 2 mM of DBF as a carbon source substrate (2.8.1) and DBF transformation was monitored over time. The cells catalyzed a rapid turnover of the substrate, with a rate of 21 µM/min. Assuming an OD of 10 to correspond to a protein content of approximately 1 g of protein per liter, this corresponds to a rate of 42 U/g protein. Degradation of DBF proceeded without accumulation of significant amounts of intermediates. To evaluate if the capability of DBF degradation was constitutive or inducible, DBF transformation rates were also quantified using cells pre-grown with fructose as probably non-inducing substrate. For this, cells pre-grown on fructose were resuspended to an OD_{600nm} of 10, incubated with 2 mM of DBF (2.8.1) and substrate depletion followed by HPLC. Substrate depletion was negligible, and < 1 µM/min. Consequently, enzymes involved in DBF degradation by HA01 are not constitutively expressed.

3.1.4 Transformation of dibenzo-p-dioxin and of chlorosubstituted dibenzofurans by *Rhodococcus* sp. strain HA01

Rhodococcus sp. strain HA01 was not capable to grow on dibenzo-p-dioxin (DD) as sole source of carbon and energy. To evaluate reasons for this failure, DBF grown resting cells (OD_{600nm} = 20) were analyzed for their capability to transform this substrate at a concentration of 1 mM. The transformation rate was only

7.2 µM/min and thus < 10 % the rate observed with DBF as substrate. It can thus be assumed that one of the reasons for the failure of HA01 to use DD as growth substrate is the poor activity of the initial dioxygenase(s) with this substrate. Transformation of DD occurred without significant accumulation of intermediates, as evidenced by HPLC analysis.

Resting cells of DBF-grown *Rhodococcus* sp. strain HA01 were capable to rapidly transform 3-chlorodibenzofuran (3CDBF, 2mM) (Fig. 3.2), and resting cells of an OD_{600nm} = 7.5, exhibited transformation rates of 8.8 µM/min which corresponds to transformation rates of 11.7 /OD_{600nm} = 10 or 28% the rate observed with DBF as substrate. HPLC analysis revealed that the substrate was not mineralized, but concomitant with the depletion of 3CDBF, accumulation of one major intermediate was observed. This intermediate showed an identical retention behavior and an identical absorption spectrum compared with an authentic standard of 4-chlorosalicylate.

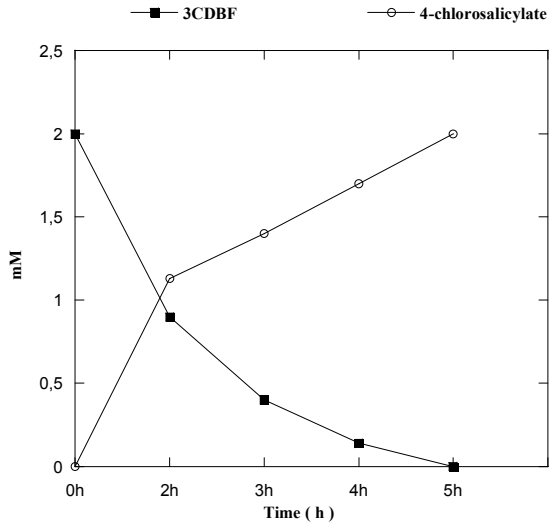

Fig. 3.2. Transformation of 3-chlorodibenzofuran (3CDBF, 2mM) by resting cells of *Rhodococcus* sp. strain HA01 of an OD_{600nm}=7.5. Substrate depletion and product accumulation were quantified by HPLC analysis.

Assuming identity of the metabolite with 4-chlorosalicylate, its quantity could be calculated by comparison with an authentic standard. Such comparison indicated quantitative transformation of 3-chlorodibenzofuran into 4-chlorosalicylate. However, the culture supernatant during 3-chlorodibenzofuran transformation showed a yellow coloration, which is indicative for the formation of an extradiol ring-cleavage product. The yellow coloration was not depleted during further incubation, indicating this compound to be a dead-end metabolite, probably formed by lateral dioxygenation of 3CDBF followed by dehydrogenation and ring-cleavage. Spectrophotometric analysis of the culture supernatant after complete transformation of 3CDBF showed the yellow product to exhibit an absorption maximum at 455 nm (Fig. 3.3) similar to the reported absorption maximum of the *meta*-cleavage product

of 1,2-dihydroxydibenzofuran (464 – 470 nm (Seeger *et al.*, 2001; Selifonov *et al.*, 1991)). As *meta*-cleavage products usually exhibit extinction coefficients > 10,000 $M^{-1}cm^{-1}$ (Happe *et al.*, 1993; McKay *et al.*, 2003) and the extinction coefficient of the extradiol cleavage product of 1,2-dihydroxydibenzofuran can be calculated to have an extinction coefficient of 34,000 $M^{-1}cm^{-1}$, it can be assumed that the absorption A_{455nm} of < 1 indicates the accumulation of < 0.1 mM of product. Thus, the amount of extradiol cleavage product accumulating corresponds to less then 5% of substrate applied.

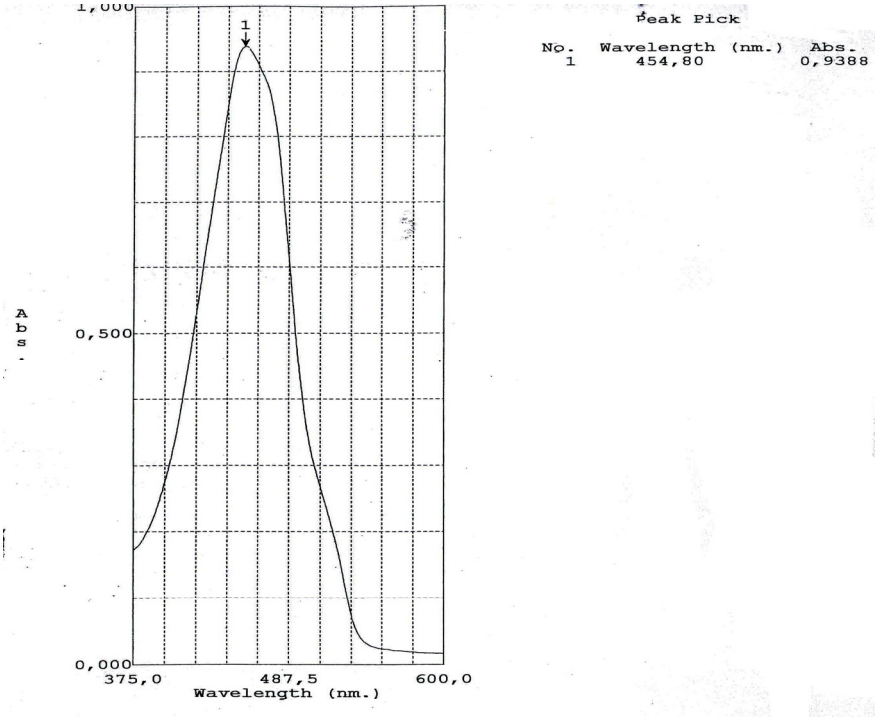

Fig. 3.3. Absorption spectrum of cell free culture supernatant after complete transformation of 2 mM 3-chlorodibenzofuran by *Rhodococcus* sp. strain HA01.

To confirm the identity of the major product formed from 3CDBF with 4-chlorosalicylate, HPLC/MS analysis was performed in the negative ionization mode. The major metabolite exhibited a molecular ion at m/z = 171 (Fig. 3.4), which is in agreement with the molecular mass of 4-chlorosalicylate of 172. The presence of an ion at m/z = 173 of about 30% the intensity of the ion at m/z = 171 confirms the parent compound to contain one chlorosubstituent. The absorption spectrum of the metabolite shown in Fig. 3.5 is moreover identical to that of authentic 4-chlorosalicylate. Consequently, it can be assumed that the major metabolite formed from 3-chlorodibenzofuran is 4-chlorosalicylate.

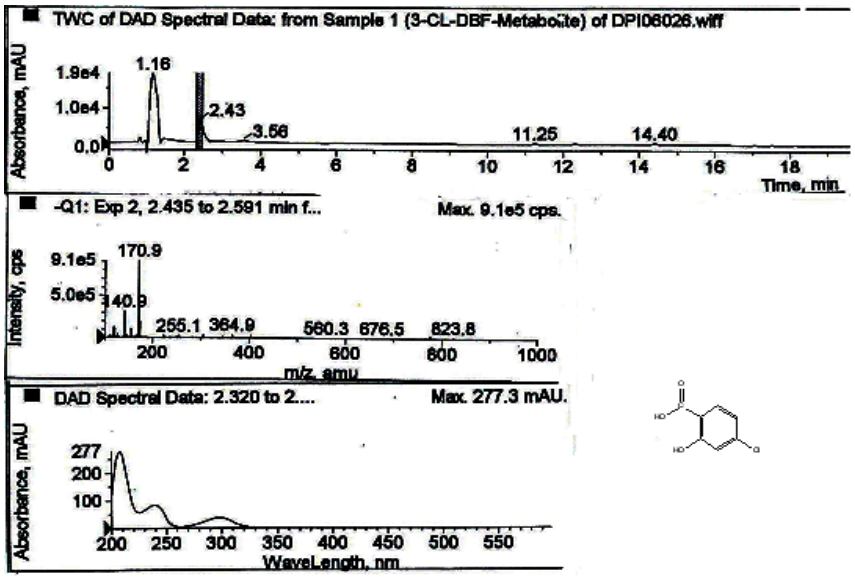

Fig. 3.4. Identification by HPLC/MS of 4-chlorosalicylate as major metabolite formed from 3-chlorodibenzofuran by *Rhodococcus* sp. strain HA01. Shown are (from top to bottom) the total wavelength chromatogram (TWC), the mass spectrum (negative ionization mode) of the metabolite eluting after 2.4 min and the UV-spectrum.

3.1.5 Degradation of 2-chlorodibenzofuran

The transformation of 2-chlorodibenzofuran (2CDBF) (2mM) by DBF-grown cells of *Rhodococcus* sp. strain HA01 occurred at rates much lower than those observed with 3CDBF. Cells of an OD_{600nm} = 16, showed transformation rates of 7.2 µM/min, which corresponds to a transformation rate of 4.5 µM/min of cells of an OD_{600} = 10, and 11% and 38% the transformation rates observed with DBF and 3CDBF, respectively. Three metabolites were detected by HPLC (Fig. 3.5).

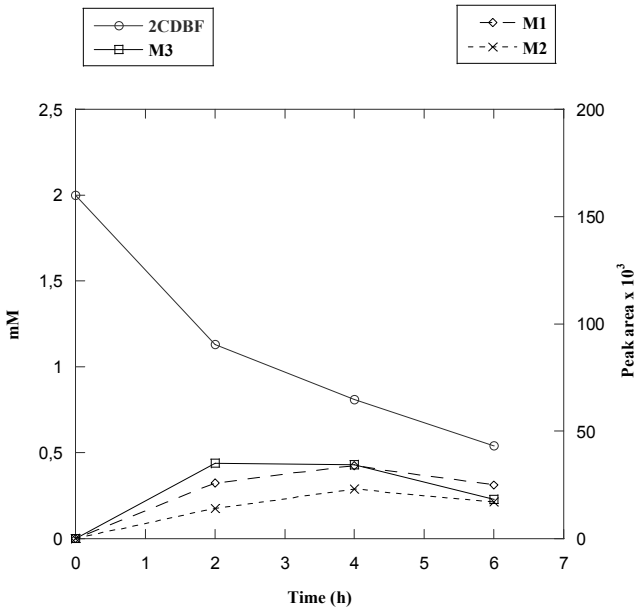

Fig. 3.5. Transformation of 2-chlorodibenzofuran (2CDBF, 2 mM) by resting cells of *Rhodococcus* sp. strain HA01 of an OD_{600nm}= 16. Substrate depletion and product accumulation were quantified by HPLC analysis. The concentrations of 2CDBF and 5-chlorosalicylate (M3) are given in mM whereas the amounts of metabolites accumulating (M1, M2 as well as M3 = 5-chlorosalicylate) as indicated by the peak area (210 nM) quantified by HPLC analysis.

One of the metabolites (M3) was identified as 5-chlorosalicylate based on the identical retention behavior and UV spectrum compared with an authentic standard. Moreover, HPLC/MS confirmed the identity of M3 with 5-chlorosalicylate (Fig. 3.6).

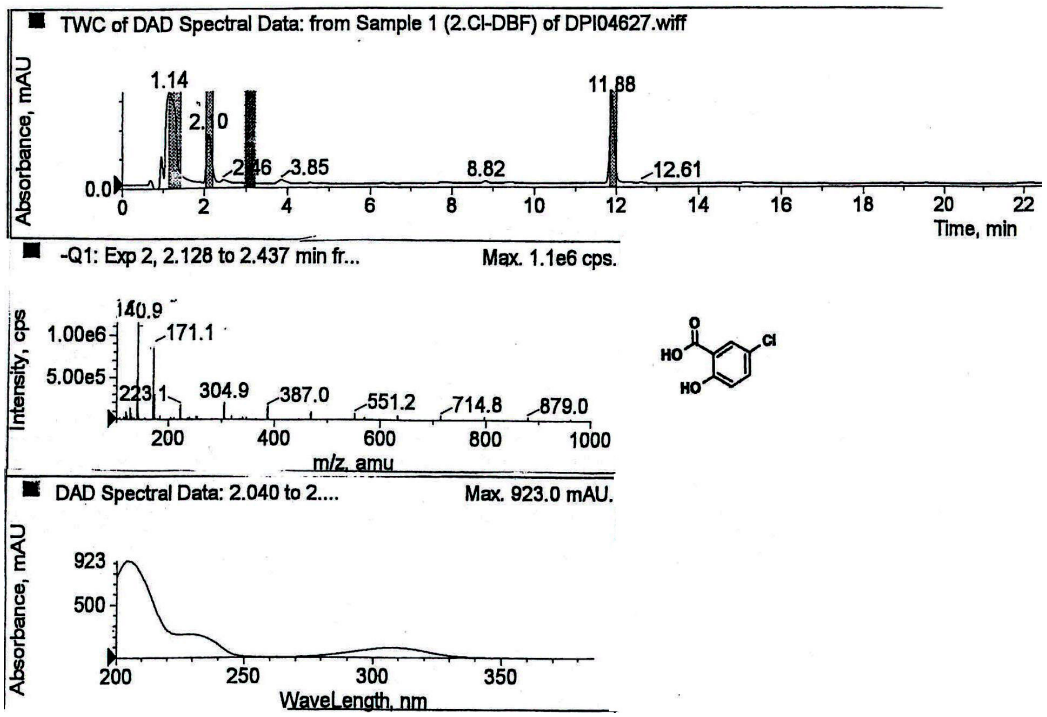

Fig. 3.6. Identification by HPLC/MS of 5-chlorosalicylate as metabolite formed from 2-chlorodibenzofuran by *Rhodococcus* sp. strain HA01. Shown are (from top to bottom) the total wavelength chromatogram (TWC), the mass spectrum (negative ionization mode) of the metabolite eluting after 2.4 min and the UV-spectrum.

Quantification with an authentic standard revealed that 35-45% of the applied 2-chlorodibenzofuran was transformed into 5-chlorosalicylate. Metabolite 1 (M1) could be identified based on its characteristic absorption spectrum to elute with a retention volume of 11,9 ml during gradient elution by HPLC/MS. MS indicated M1 to have a molecular ion of (negative ionization) m/z=235/237 (Fig. 3.7) showing the parent compound to have a $C_{12}H_9ClO_3$ composition. This indicates M1 to be either a chlorosubstituted trihydroxybiphenyl or a dihydrodiol of 2CDBF. Metabolite M2 was obviously formed only in minor amounts when incubation was performed for up to 2 hours.

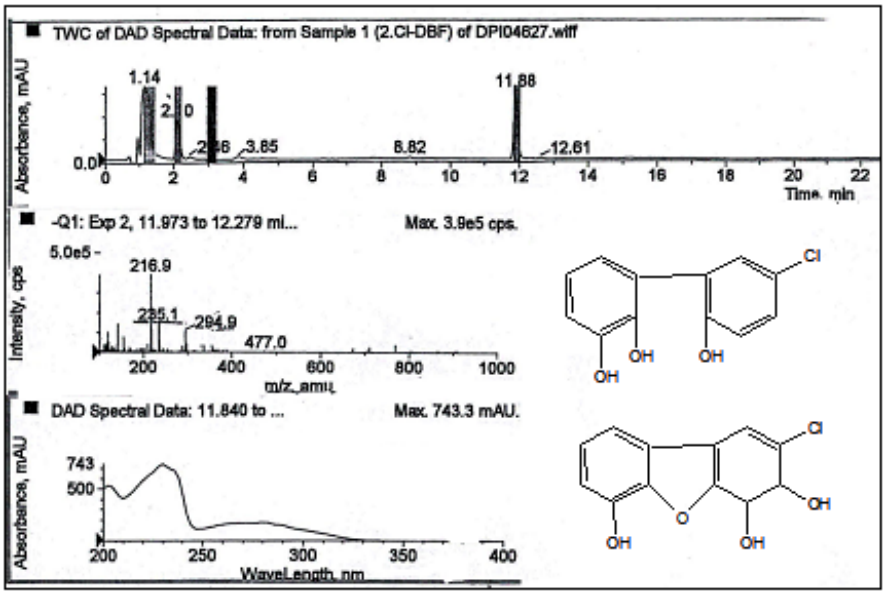

Fig. 3.7. Identification by HPLC/MS of the metabolite formed from 2-chlorodibenzofuran by *Rhodococcus* sp. strain HA01 to have a $C_{12}H_9ClO_3$ composition. Shown are (from top to bottom) the total wavelength chromatogram (TWC), the mass spectrum (negative ionization mode). of the metabolite eluting after 11.9 min and the UV-spectrum.

To identify the structure of M1, which is obviously formed in major amounts, a freshly prepared product mixture containing mainly M1 and M3 was subject to in-situ ^{1}H NMR analysis (Fig. 3.8). This analysis showed the presence of two major metabolites, with one of the metabolites being 5-chlorosalicylate. Three aromatic protons of this compound showed chemical shifts of δ= 6.96 (H-3), 7.45 (H-4) and 7.83 (H-6) ppm respectively. Coupling constants of J (H-3/H-4) = 9.0 Hz and J (H-4/H-6)= 2.2 Hz are in agreement with the supposed structure.

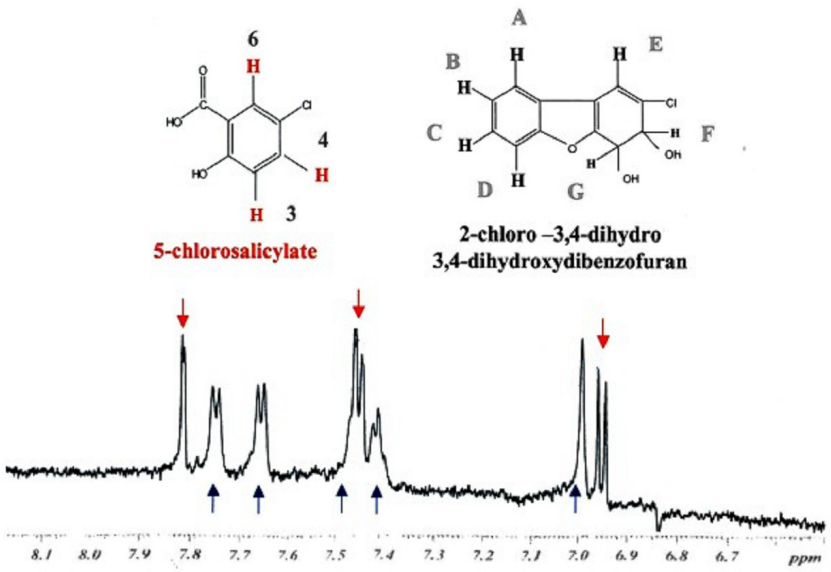

Fig. 3.8. ^{1}H NMR spectrum of the metabolite mixture formed from 2CDBF indicate the presence of two major metabolites in approximately equal amounts. One of them is identical with 5-chlorosalicylate. The NMR of the second metabolite indicates its identity with 2-chloro-3,4-dihydro-3,4-dihydroxydibenzofuran.

The second metabolite evidently comprised five aromatic or vinylic protons, exhibiting chemical shifts of δ = 7.75(H-A), 7.42 (H-B), 7.45 (H-C), 7.67 (H-D) and 6.99 (H-E) ppm, respectively. Even thought the signals of H-4 of 5-chlorosalicylate and of H-C of the second metabolite could not be separated, the integral of the resonance lines clearly showed two distinct protons resonating at 7.45 ppm. The presence of five aromatic or vinylic protons excludes the identity of metabolite 1 with a chlorosubstituted trihydroxybiphenyl (either 2,2´3-trihydroxy-5-chlorobiphenyl or either 2,2´3-trihydroxy-5-chlorobiphenyl can be formed after angular dioxygenation), which harbour 6 aromatic protons and indicates metabolite 1 to be a chlorosubstituted DBF dihydrodiol. As the metabolite evidently contained at least four aromatic protons, showing vicinal couplings (J = 8 - 10 Hz) with at least one other proton, dioxygenolytic attack should have occurred at the chlorosubstituted ring. Thus, the observed NMR data are in accordance with the structure of metabolite 1 as 2-chloro-3,4-dihydro-3,4-dihydroxydibenzofuran, with a vinylic proton resonating at δ = 6.99 ppm (H-E). Protons F and G (see Fig. 3.8), are expected to resonate at δ = 4 – 4.5 ppm and are probably covered by the suppressed water signal. Comparison of the integrals of the resonance lines showed that in different preparations, the ratio in which these products have been formed (5-chlorosalicylate: 2-chloro-3,4-dihydro-3,4-dihydroxydibenzofuran) was always 0.8-0.9:1. No indications for the significant accumulation of a third metabolite were observed.

3.1.6 Transformation DBF, DD, 3CDBF, and 2CDBF in the presence of 3-chlorocatechol

3-Chlorocatechol is known as a strong inhibitor of extradiol dioxygenases (Bartels *et al.*, 1984; Vaillancourt *et al.*, 2002). It has thus been used in experiments with *Sphingomonas wittichii* RW1 to elucidate regioselectivity of initial dioxygenation (Wittich *et al.*, 1992), as the ring-cleavage substrate intermediates tend to accumulate in the presence of 3-chlorocatechol. In fact, one intermediate was observed to accumulate when DBF-grown cells of *Rhodococcus* sp strain HA01 (OD_{600nm} = 10) were incubated with 1.0 mM DBF and 0.1 mM 3-chlorocatechol. The metabolite, which was not observed when the same cells were incubated in the absence of 3-chlorocatechol, exhibited a retention behavior during reserved phase HPLC identical to that of authentic 2,2',3-trihydroxybiphenyl (THB). However, inhibition of ring-cleavage activity was not complete, and only up to 20% of the substrate applied accumulated as THB. During extended incubation, THB was further degraded (Fig. 3.9). Overall, the transformation rate of DBF was significantly influenced by the presence of 3-chlorocatechol, and accounted for only 20% that observed in the absence of 3-chlorocatechol.

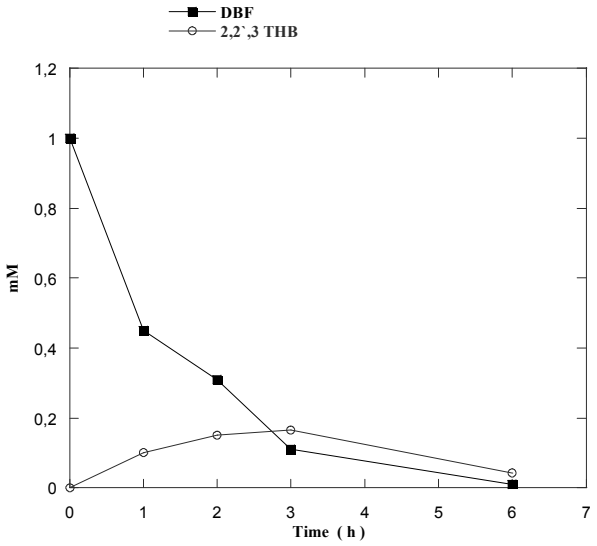

Fig. 3.9. Transformation of DBF (1 mM) in the presence of 3-chlorocatechol (0.1 mM) by DBF-grown cells of *Rhodococcus* sp. strain HA01 (OD_{600nm} =10) as followed by HPLC analysis.

When DBF-grown resting cells (OD_{600nm} = 20) were incubated with DD (1 mM) and 0.1 mM 3-chlorocatechol as inhibitor, DD was slowly depleted from the reaction mixture (Fig. 3.10) with rates only 20% those observed in the absence of inhibitor. The intermediate accumulation of one metabolite was observed, which cochromatographed by HPLC analysis with authentic THBE, and showed an identical UV-spectrum. HPLC/MS revealed both THBE and the formed metabolite to exhibit an [M-H] molecular ion of m/z=217, when analysis was performed in the negative ionization mode, in accordance with the THBE $C_{12}H_{10}O_4$ structure (Fig. 3.11).

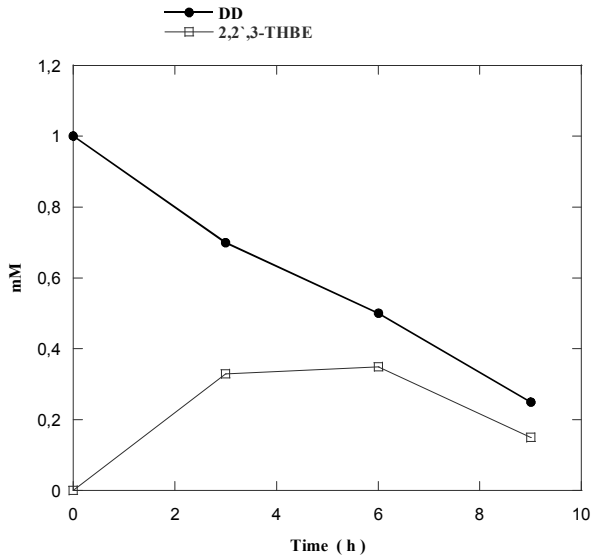

Fig. 3.10. Transformation of DD (1 mM) in the presence of 3-chlorochatechol (0.1 mM) by DBF-grown cells of *Rhodococcus* sp. strain HA01 (OD_{600nm} =20) as followed by HPLC analysis.

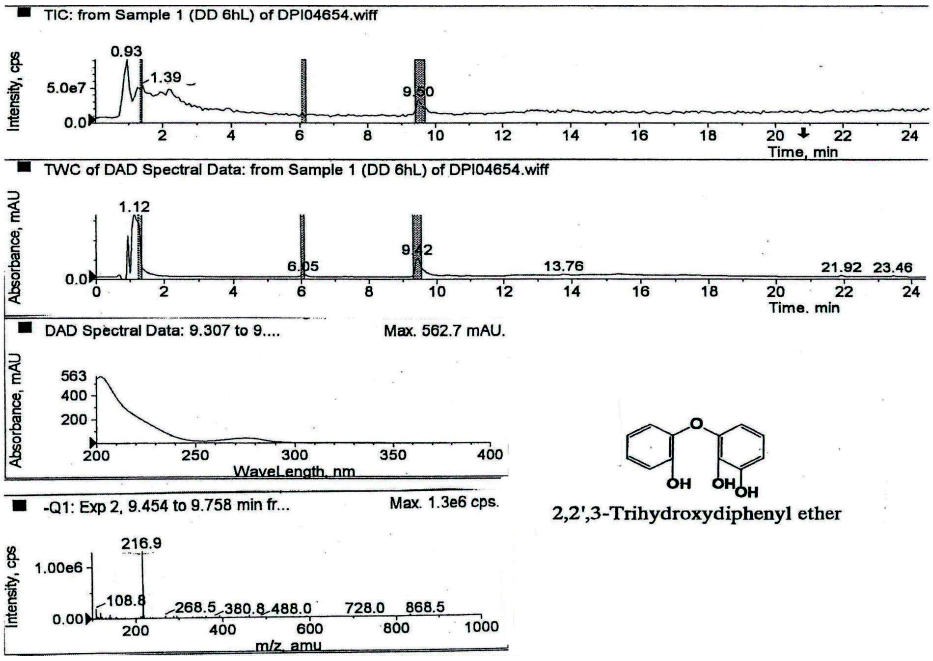

Fig. 3.11. Identification by HPLC/MS of THBE as metabolite of DD transformation by *Rhodococcus* sp. strain HA01. Shown are (from top to bottom) the total ion current (TIC), the total wavelength chromatogram (TWC), the UV-spectrum of the metabolite eluting after 9.4 min and the mass spectrum (negative ionization mode).

However, THBE accumulation was not quantitative and accounted for less than 40% of the substrate applied, indicating ring-cleavage activities to be not quantitively inhibited by 3-chlorocatechol. In accordance with only partial inhibition of ring-cleavage activities, THBE was further transformed at low rates during the further course of the experiment.

When DBF-grown cells (OD_{600nm} = 10) were incubated with 3CDBF and 3-chlorochatechol as an extradiol dioxygenase inhibitor, like in the cases described above, the transformation rate was significantly reduced as compared to the experiment without inhibitor. HPLC analysis showed the accumulation of 4-chlorosalicylate as major and only metabolite as observed in the experiment without inhibitor. No indications for the significant accumulation of hydroxylated intermediates were observed. This shows that, despite the significant inactivation of ring-cleavage activities by 3-chlorocatechol revealed in experiments with DBF and DD as substrates ring-cleavage of 2,2', 3- trihydroxy-4'-chlorodibenzofuran, the putative precursor of 4-chlorosalicylate, was not the bottleneck in this transformation. Analysis of the presence of 3-chlorocatechol on the transformation of 2CDBF by DBF-grown cells (OD_{600nm} = 16) showed, as indicated above, only a significant reduction in the transformation rate. However, the metabolite profile did not significantly differ from that observed in the absence of 3-chlorocatechol.

Table 3.1. Transformation of DBF, DD and chlorinated dibenzofurans by DBF and fructose grown cells of *Rhodococcus* sp. strain HA01. Activities given are those standardized to an OD_{600nm} of 10.

Growth substrate	Substrate	Activity (µM/min) in the absence of 3-chlorocatechol	Activity (µM/min) in the presence of 3-chlorocatechol
Fructose	DBF	2	ND
DBF	DBF	43	10
DBF	DD	4	1
DBF	3CDBF	13	3
DBF	2CDBF	5	2

3.1.7 Genetic analysis of *Rhodococcus* sp. strain HA01

3.1.7.1 PCR amplification and characterization of genes encoding a Rieske non heme iron oxygenase in *Rhodococcus* sp. strain HA01

As described above, strain HA01 showed a regioselectivity of attack on 2CDBF not previously reported. As moreover, only a few genes involved in the metabolism of DBF via angular dioxygenation had been described, it was attempted to identify genes responsible for DBF angular dioxygenation in HA01. Two sets of degenerated primers, RieskeF and RieskeR (Kasuga *et al.*, 2001) and DOα-2 and DOα-3 (Iida *et al.*, 2002a) had previously been designed for the amplification of fragments of genes encoding Rieske non-heme iron oxygenases and had successfully been applied for amplification of angular dioxygenase gene fragments (Iida *et al.*, 2002a; Kasuga *et al.*, 2001). RieskeF and RieskeR had been designed to amplify a short 78 bp fragment comprising the highly conserved region encoding the Rieske 2Fe-2S cluster binding sites localized on the α-subunits of the terminal oxygenases involved in the degradation of polycyclic aromatics. Also the degenerate PCR primers DOα-2 and DOα-3 (Iida *et al.*, 2002a) have been designed to anneal to nucleotides encoding conserved amino acid sequence regions of the α-subunits of Rieske non-heme iron oxygenases. When these primers were used under the published PCR conditions (Iida *et al.*, 2002a; Kasuga *et al.*, 2001) for amplifying DNA fragments probably encoding part of the angular dioxygenase of *Rhodococcus* sp. strain HA01, no products were observed in either case. Gradient PCR was used for the two sets of primers (2.10.2.2.2) and the expected amplification product of 78 bp fragment was observed using the RieskeF and RieskeR primers and a PCR amplification product of the expected 70 bp size was observed when using the DOα-2 and DOα-3 primers. The obtained PCR products were ligated into pGEM®-T Easy Vector (2.10.7) and transformed into *E. coli* JM109 after heat shock (2.10.9.1). After plating, different colonies were analyzed by colony PCR (2.10.8) using universal M13 forward and reverse primers. PCR products showing the presence of inserts of the expected size (Fig. 3.12) were purified. Sequence analysis of inserts (2.10.5) showed that the DOα-2 and DOα-3 failed to amplify fragments of genes encoding oxygenases in HA01. Analysis of eight PCR products obtained with the RieskeF/RieskeR primer set revealed the presence of two different dioxygenase gene fragments. One of those fragments present in two of the analyzed PCR products showed the highest sequence identity (92%) to gene fragments encoding the Rieske clusters of benzoate dioxygenases (Fig. 3.13, lane 3 and 8).

The second fragment present in four of the analyzed PCR products was highly homologous (99%) to the gene fragment of the α-subunit of the DfdA angular dioxygenase of *Terrabacter* sp. strain YK3 (Fig. 3.12 lane 4, 5, 6, and 7).

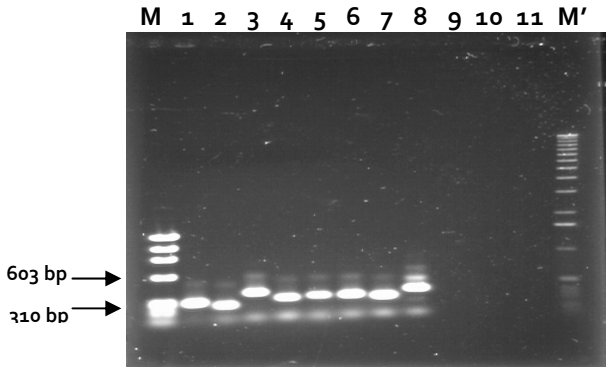

Fig. 3.12. Agarose gel electrophoresis of PCR products obtained using M13 primers on *E.*coli JM109 colonies containing pGEM®-T Easy Vector with inserts obtained by amplification using the RieskeF/RieskeR primer set (lane 1-8). Lanes M and M': molecular weight markers IX and X (Roche), respectively.

To obtain more sequence information of the gene putatively encoding an angular dioxygenase in HA01, the obtained sequence was used to design two specific forward primers (YdbfDOF1 and YdbfDOF2). Degenerated reverse primers were designed based on conserved regions of genes encoding ferredoxin reductases involved in the degradation of polycyclic aromatic hydrocarbons (PAHs), which are often localized downstream of genes encoding the α-subunits of Rieske non-heme iron oxygenases. As the resultant multiple alignment of the amino acid sequences of ferredoxin reductases revealed the presence of two conserved regions, two reverse degenerated primers (HamdyR1 and HamdyR2) were designed. Gradient PCR (2.10.2.2.2) was used with all four combinations of forward (YdbfDOF1 and YdbfDOF2) and reverse (HamdyR1 and HamdyR2) primers. PCR products were only obtained using YdbfDOF1 and HamdyR1 primers. As PCR products of different length were obtained, touchdown PCR (2.10.2.2.2) was applied, which resulted in a single PCR product of 2.5 Kb (Fig. 3.13), which was purified and sequenced (2.10.3, 2.10.4, and 2.10.5) using the same primers.

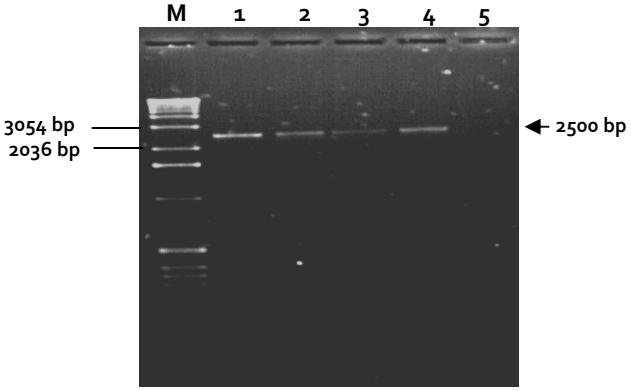

Fig. 3.13. Agarose gel electrophoresis of the PCR product obtained using YdbfDOF1 forward and HamdyR1 reverse primers (lane 1- 4) on genomic DNA of *Rhodococcus* sp. strain HA01. Lane M, molecular weight markers X (Roche); lane 5, control reaction comprising only the primers but no template DNA.

The single read of the sequence obtained with the YdbfDOF1 primer exhibited a high similarity with the *dfdA* gene of *Terrabacter* sp. strain YK3 encoding the DfdA α-subunit of dibenzofuran dioxygenase. The sequence read obtained with primer HamdyR1 consistently showed a high similarity with the target intended, and specifically with ferredoxin reductase and ferredoxin encoding *dfdA3* and *dfdA4* genes of *Terrabacter* sp. strain YK3. Specific forward and reverse primers (AlphaF2S, and FerredoxinR1S) annealing at the borders of the obtained sequence were designed in order to resolve more inner sequence of this 2.5 Kb fragment in both directions, and to analyze if in fact a gene cluster fragment highly similar to the *dfdA1A2A3A4* gene cluster in *Terrabacter* sp. strain YK3 was obtained.

For this purpose, primers (Table 2.4) were successively designed to obtain the complete sequence of the fragment on both strands.Taking in account the high similarity of the obtained sequence of the whole cluster fragment with the *dfdA1A2A3A4* gene cluster of *Terrabacter* sp. strain YK3 (GenBank accession number AB075242) encoding the α-and β subunits, ferredoxin reductase and ferredoxin components of the dibenzofuran dioxygenase, a primer set was designed (F546and R4900) to amplify the complete gene cluster mentioned above, but using as template *Rhodococcus* sp. HA01 genomic DNA. A PCR product of the expected 4.4 Kb (Fig. 3.14) was obtained. Several oligonucleotides were designed to perform the primer walking and sequence assemblage (2.10.2.2.1).

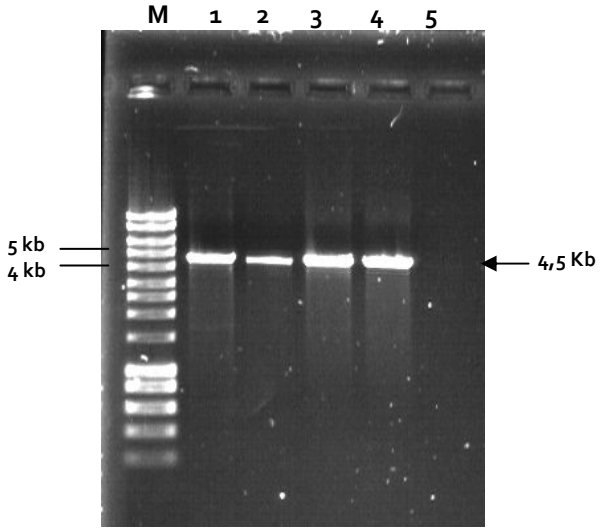

Fig. 3.14. Agarose gel electrophoresis of the 4,4 kb product obtained using the F546 and R4900 primer set on genomic DNA of *Rhodococcus* sp. strain HA01 (lane 1-4). Lane M, molecular weight markers X (Roche); lane 5, PCR negative control reaction (no template DNA).

High similarity over the whole sequence was observed with the *dfdA1A2A3A4* gene cluster of *Terrabacter* sp. strain YK3 (Iida *et al.*, 2002a). The obtained sequence contained about 250 bp of non-coding sequence upstream of the predicted start codon of the *dfdA1* gene of HA01 and 150 bp downstream of the stop codon of the *dfdA4* ferredoxin reductase encoding gene. The predicted *dfdA1* α- subunit differed from the one of *Terrabacter* sp. strain YK3 by two amino acids, whereas the predicted *dfdA2* β subunits of YK3 and HA01 were identical. Three amino acid differences were observed between the DfdA3 ferredoxins and the DfdA4 ferredoxin reductase of HA01 was five amino acids longer compared to DfdA4 of YK3.

3.1.8 Expression of *dfdA* in *Rhodococcus* sp. strain HA01

To characterize if the identified *dfdA* genes are expressed in response to DBF, RT-PCR experiments were performed with total RNA extracted from *Rhodococcus* sp. strain HA01 growing on DBF or fructose. Amplification products of the expected size were observed in the culture grown on DBF using the FYK3RNA/RYK3RNA primers set targeting the α-subunit (DfdA1) whereas no product was detected with RNA extracted from the fructose grown culture (Fig. 3.15). In addition, no amplification products were observed in controls devoid of reverse transcriptase or template cDNA (Fig. 3.15 A, B, lanes 15 - 18 and C, lanes 1- 5). Sequencing of the approximately 435 bp product confirmed that it was identical to the corresponding *dfdA* gene fragment from genomic DNA of *Rhodococcus* sp. strain HA01 (Fig. 3.15 C lane 6′). These results showed that the DfdA dioxygenase from *Rhodococcus* sp. strain HA01 is specifically induced in the presence of DBF.

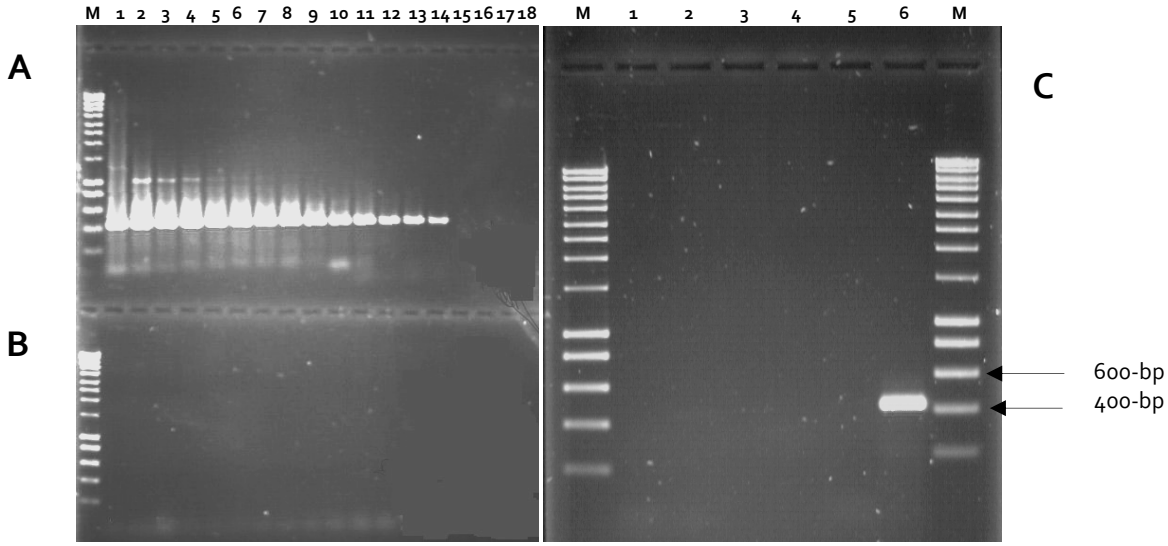

Fig. 3.15. RT-PCR amplification of *dfdA*-mRNA from *Rhodococcus* sp. strain HA01 grown on DBF (Panel A) or fructose (Panel B). M, molecular weight marker Hyperladder 1 (Bioline). cDNA generated from template RNA was serially diluted (3.2-fold) with nuclease-free water and 1 µl of each dilution was subjected to amplification by PCR (Lanes 1 - 14). Negative controls included RT and PCR reactions devoid of reverse transcriptase (lane 15 - 17 of panel A and B and lane 1 - 4 of panel C) and template cDNA (lane 18 of panel A and B and lane 5 of panel C), respectively. PCR reactions containing 1 ng of genomic DNA as a template were used as positive control (Panel C, lane 6).

3.1.9 Heterologous expression of DfdA dioxygenase from *Rhodococcus* sp. strain HA01.

3.1.9.1 Expression in *E. coli* JM109

The complete gene cluster comprising the *dfdA1A2A3A4* genes probably encoding a dibenzofuran dioxygenase consisting of the α-and β subunits, ferredoxin and ferredoxin reductase was amplified from DNA of *Rhodococcus* sp. strain HA01 by use of the primers FPST1yk3/RECOyk3, which include artificial restriction sites for *EcoR1* and *Pst1* restriction enzymes, and annealed 240 bp upstream and 137 bp downstream, respectively, of the complete *dfdA1A2A3A4* gene cluster. The obtained fragment was

digested by *EcoR1* and *Pst1* restriction enzymes and cloned into the *EcoR1* and *Pst1* sites of pUC119 to give pDFDE and introduced into *E.coli* JM109. Presence of the insert was confirmed by colony PCR using two different primer sets (FYK3RNA/RYK3RNA and ferredoxinred F3000 /ferredoxinred R3500) annealing in the α-subunit and ferredoxin reductase encoding genes, respectively. *E.coli* JM109 was grown in the presence of IPTG and transformation of DBF (200 µM) was tested by resting cells of an OD_{600nm} of up to 10. However, neither the transformation of DBF nor of the formation of metabolites was observed by HPLC analysis of cell free supernatant.

3.1.9.2 Expression in *Rhodococcus* sp. ATCC 12674

As DBF transformation was obviously absent in *E.coli*, expression of the *dfdA* genes was attempted in *Rhodococcus*. *Rhodococcus* sp. ATCC 12674 was chosen as host strain. This strain was verified not to be capable to degrade DBF. The *Rhodococcus-E.coli* shuttle vector pRSG43 (Fig. 3.16), which had been constructed by Masahiro Takeo, was used for cloning. As described above, the complete gene cluster comprising the *dfdA1A2A3A4* genes of HA01 was amplified by primers (FPST1yk3/RECOyk3) after digestion with *Pst1* and *EcoR1* restriction enzymes, cloned into the *EcoR1* and *Pst1* sites of pRSG43 to give pDFDR, and introduced into *Rhodococcus* sp. ATCC 12674 by electroporation (2.10.9.3). At the same time, as a negative control, *Rhodococcus* sp. ATCC 12674 was transformed with the shuttle vector pRSG43 without insert. Presence of the 4.4 kb insert in pDFDR was confirmed by colony PCR using the primer sets FYK3RNA/RYK3RNA and ferredoxinredF3000/ferredoxinredR3500. PCR products of the expected length were obtained from clones containing pDFDR whereas PCR products were absent when clones containing pRSG43 were analyzed.

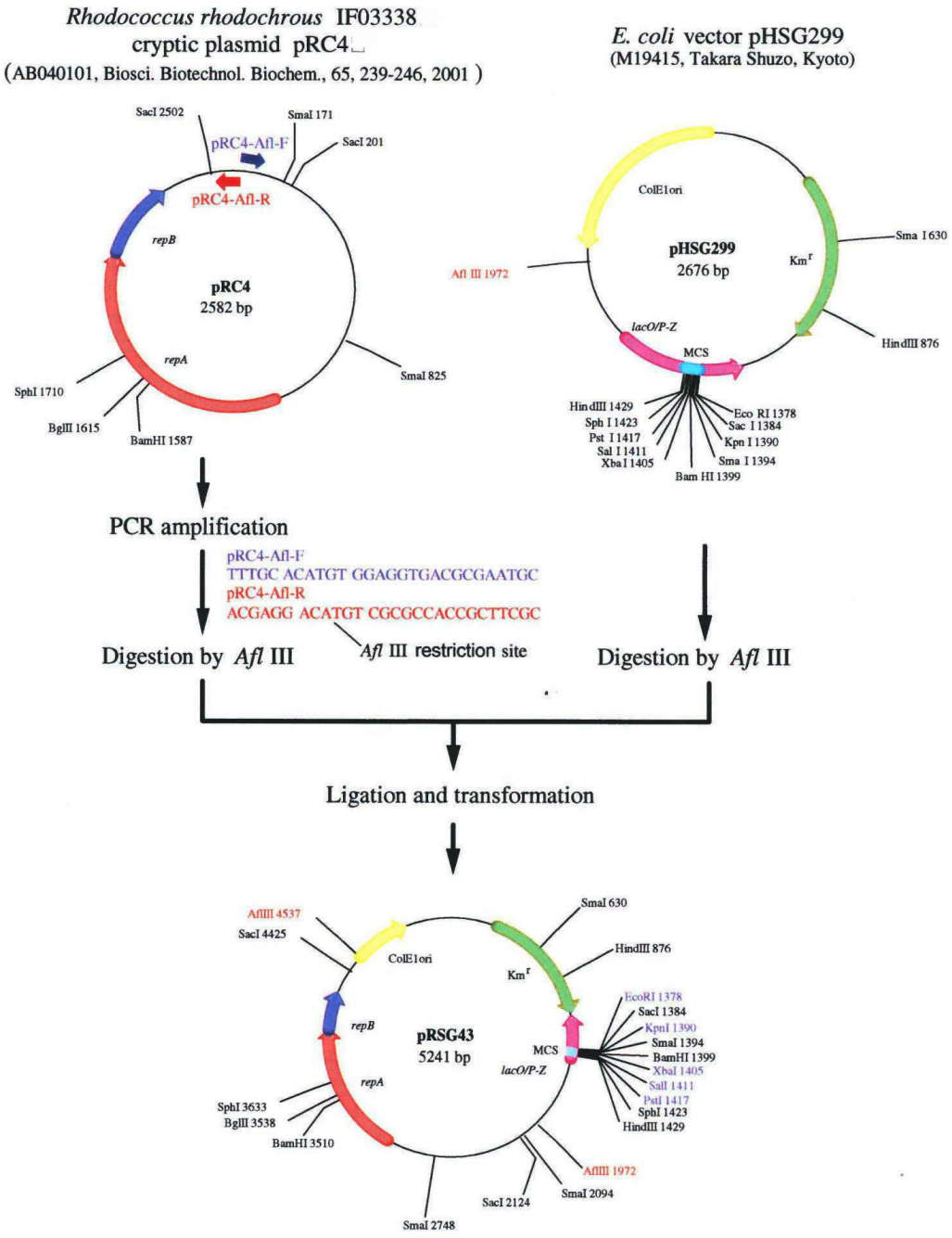

Fig. 3.16. Construction of the *Rhodococcus-E.coli* shuttle vector pRSG43 as performed by Masahiro Takeo. Unique restriction sites in the MCS are shown in blue color.

3.1.10 Analysis of *dfdA* expression by SDS PAGE

To analyze whether the *dfdA* genes on pDFDR were expressed and translated to polypeptides with the predicted sizes in *Rhodococcus* sp. ATCC 12674, cells haboring pRSG43 and cells harboring pDFDR were grown in the presence and absence of IPTG and cell extracts compared by SDS-PAGE (2.8.7). As shown in Fig. 3.17, a prominent band of a molecular mass of approximately ~51 kDa was observed in cell extracts of *Rhodococcus* sp. ATCC 12674 (pDFDR), which was absent in cell extracts of *Rhodococcus* sp. ATCC 12674

(pRSG43). This is in agreement with the expected molecular mass of the *dfdA1* gene product (53 kDa). IPTG obviously did not effect *dfdA* expression.

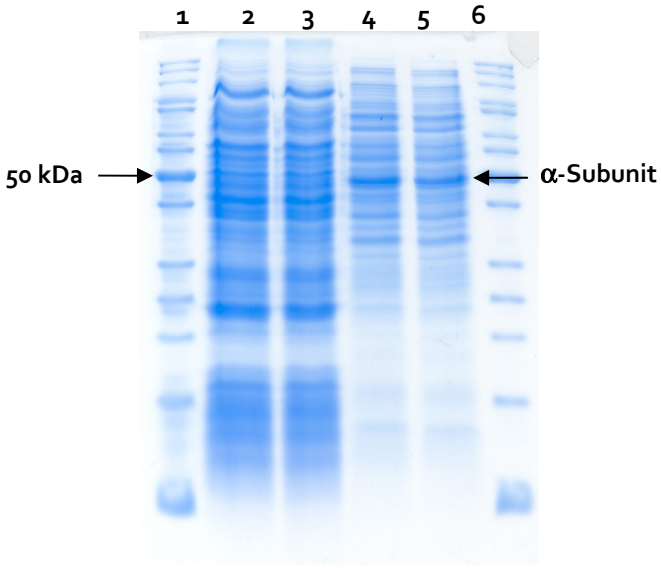

Fig. 3.17. SDS-PAGE analysis of cell extracts of *Rhodococcus* sp. ATCC 12674 (lane 2), *Rhodococcus* sp. ATCC 12674 (pRSG43) (lane 3), *Rhodococcus* sp. ATCC 12674 (pDFDR) pregrown in the presence of IPTG (lane 4) and *Rhodococcus* ATCC 12674 (pDFDR) grown in the absence of IPTG (lane 5). 10 µl (lane 2 and 3) or 5 µl (lane 4 and 5) of extracts were subjected to SDS-PAGE. Lane 1 and 6, Page Ruler unstained protein ladder (MBI Fermentas).

3.1.11 Transformation of DBF, 3CDBF, 2CDBF, DD and carbazole by *Rhodococcus* sp. ATCC 12674 (pDFDR)

To analyze if an active angular dioxygenase was present in cells of *Rhodococcus* sp. ATCC 12674 (pDFDR), resting cells were prepared and the transformation of 0.5 mM DBF was evaluated using an OD_{600nm} = 10. Whereas cells of *Rhodococcus* sp. ATCC 12674 (pRSG43) were not capable of DBF transformation and no metabolites were observed by HPLC analysis, cells of *Rhodococcus* sp. ATCC 12674 (pDFDR), were capable of DBF transformation with a rate of 1 µM/min (Fig. 3.18). A single metabolite was observed, which cochromatographed with authentic THB and showed an identical UV-absorption spectrum. Transformation of DBF into THB was quantitative, confirming that the *dfdA1A2A3A4* genes from *Rhodococcus* sp. strain HA01 encode for a functional angular dioxygenase.

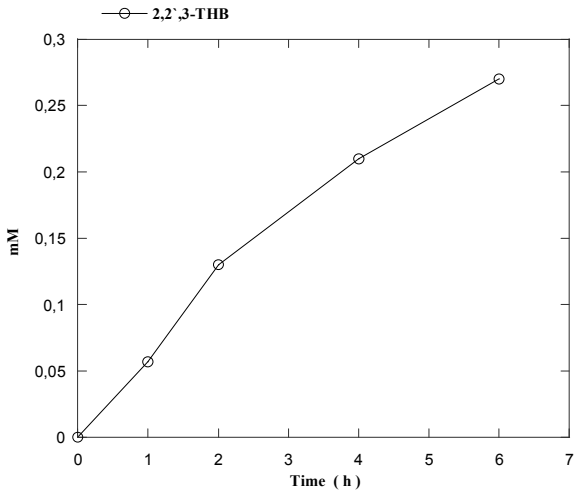

Fig. 3.18. Transformation of DBF (0.5 mM) into THB by *Rhodococcus* sp. ATCC 12674 (pDFDR) (OD_{600nm}=10) as followed by HPLC analysis.

Resting cells of *Rhodococcus* ATCC 12674 (pDFDR) also converted 3CDBF into one product. As evidenced by HPLC analysis, 3CDBF (applied at a concentration 0.5 mM) was transformed by cells of an OD_{600nm} = 10 at a rate of 0.29 µM/min (Fig. 3.19). HPLC/MS revealed the formed metabolite to exhibit a [M-H] molecular ion of m/z=237 with one-third the intensity of the ion at m/z = 235 showing the metabolite to be chlorosubstituted (Fig. 3.20). This indicates the metabolite to have a ($C_{12}H_9ClO_3$) composition. As the metabolite moreover showed an UV spectrum of an aromatic metabolite, its identity with 4-chloro-2,2',3-trihydroxybiphenyl can be proposed.

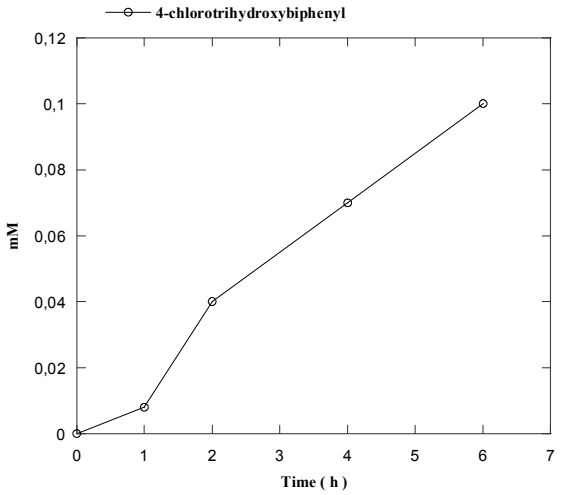

Fig. 3.19. Transformation of 3CDBF (0.5 mM) by *Rhodococcus* ATCC 12674 (pDFDR) (OD_{600nm}=10) as followed by HPLC analysis As described, the identity of the formed metabolite with 4-chloro-2,2`,3-trihydroxybiphenyl can be proposed. The concentration of 4-chloro-2,2',3-trihydroxybiphenyl formed was quantified assuming quantitative transformation of 3CDBF into this metabolite.

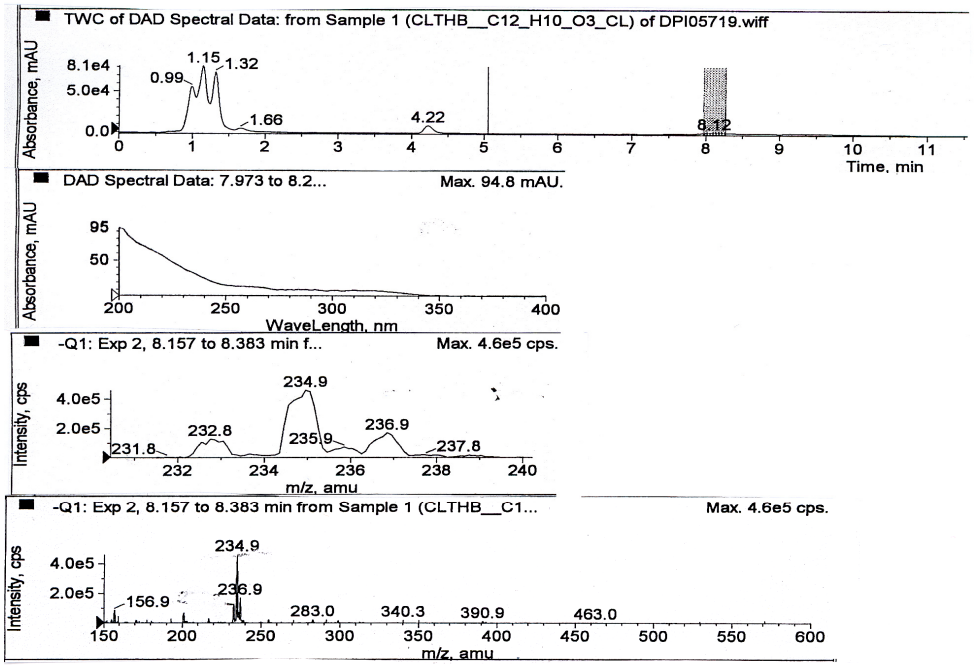

Fig. 3.20. Identification by HPLC/MS of 4-chloro-2,2',3-trihydroxybiphenyl as metabolite produced from 3CDBF by *Rhodococcus* sp. ATCC 12674 (pDFDR). Shown are (from top to bottom) the total wavelength chromatogram (TWC). The UV-spectrum of the metabolite eluting after 8.1 min and the mass spectrum (negative ionization mode).

Two metabolites were formed from DD (0.5 mM) by *Rhodococcus* sp. ATCC 12674 (pDFDR) (Fig. 3.21), which was transformed by cells of an OD_{600nm} = 10 at a rate of 0.19 µM/min. One of the metabolites, eluting under isocratic conditions at 2 min, displayed an UV spectrum with absorption maxima at 201 nm and 275 nm, and was identified as THBE by comparison of retention behavior and spectral characteristics with an authentic standard.

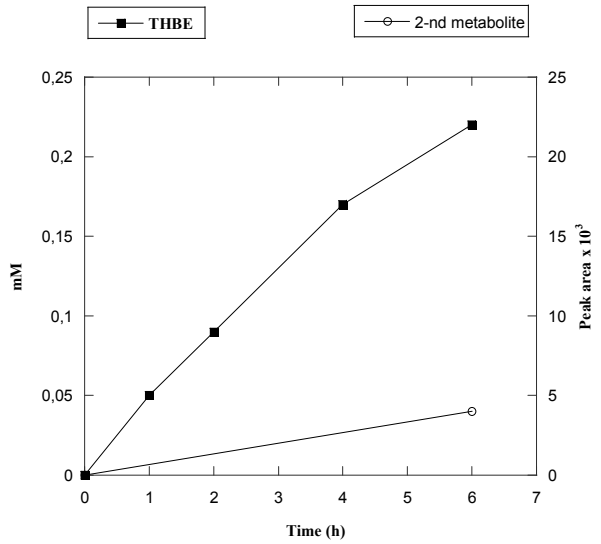

Fig. 3.21. Transformation of DD (0.5 mM) by resting cells of *Rhodococcus* sp. ATCC 12674 (pDFDR)I of an OD_{600nm} = 10. The concentrations of the product THBE is given in mM (left), but also indicated by the peak area (210 nM) quantified by HPLC analysis (right), whereas the concentration of the second metabolite, a putative dibenzo-*p*-dioxin dihydrodiol is indicated only by the peak area.

Its identity was further confirmed by HPLC/MS. Under the gradient conditions applied, THBE, like the metabolite, eluted at 7.5 min and exhibit a [M-H] molecular ion of m/ z = 217 in accordance with the THBE structure. THBE was the major metabolite formed, accounting for approximately 90% of transformed DD. The second metabolite, eluted at 2.3 min under isocratic elution conditions and at 8.08 min under the applied HPLC/MS conditions. This metabolite also displayed a [M-H] molecular ion of m/z = 217.

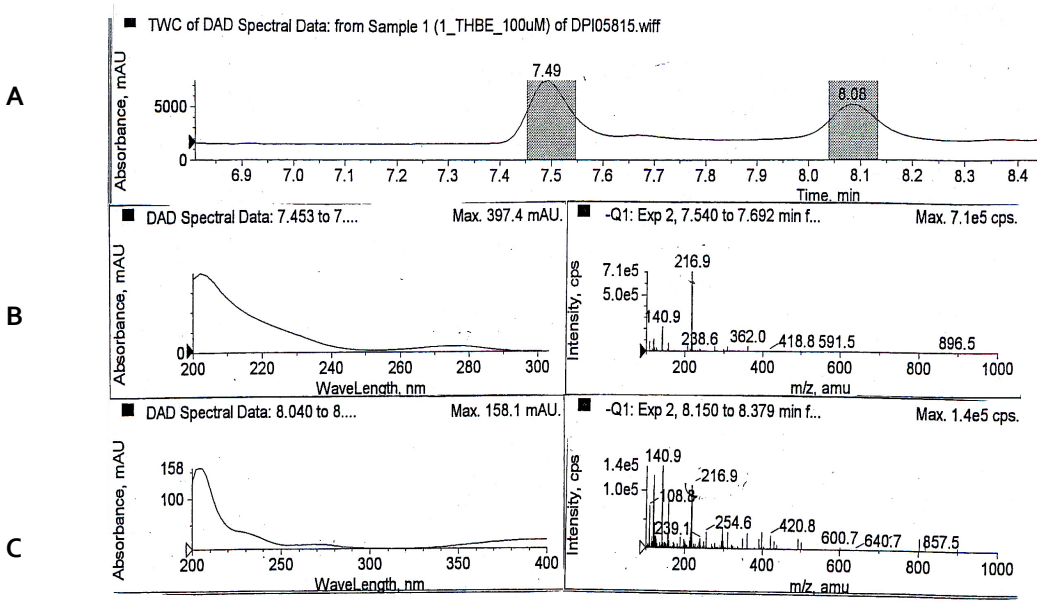

Fig. 3.22. Identification by HPLC/MS of 2,2′,3-trihydroxybiphenyl ether (lane B) and a dihydrodiol of dibenzo-*p*-dioxin (lane C) as metabolites produced from DD by *Rhodococcus* ATCC 12674 (pDFDA). Shown are the total wavelength chromatogram (TWC, lane A), the UV- spectra of the metabolites (lane B and C, left) and the mass spectrum (negative ionization mode, lane B and C, right).

Also carbazole was transformed by resting cells of *Rhodococcus* sp. ATCC 12674 (pDFDR), A single product was observed and HPLC/MS showed the metabolite to have a [M-H] molecular ion of m/z=200, indicating the metabolite to have a $C_{12}H_{11}O_2N$ composition of a carbazol dioxygenation product (Fig. 3.23). In contrast neither substrate transformation nor accumulating of metabolites were observed when *Rhodococcus* sp. ATCC 12674 (pRSG43) was used in transformation experiments with DBF, 3CDBF, DD or carbazole as a substrates. By using resting cells of Rhodococcus sp. ATCC 12674 (pDFDR) to transform 2CDBF, neither indication of the transformation of 2CDBF, nor the formation of metabolites were observed by HPLC.

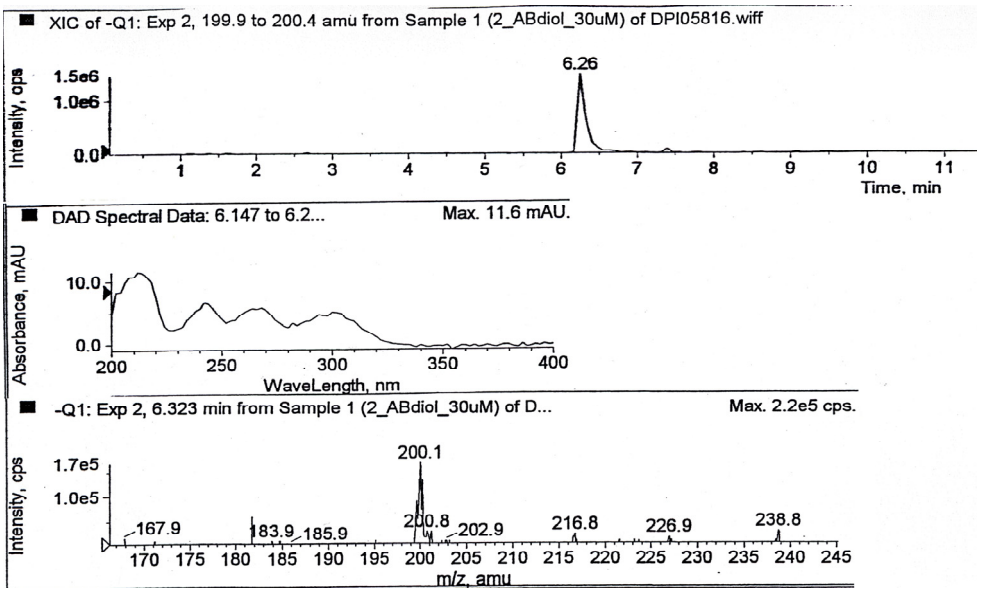

Fig. 3.23. Characterization by HPLC/MS of a metabolite, produced from carbazole by *Rhodococcus* sp. ATCC 12674 (pDFDR). Shown are (from top to bottom) the ion current (XIC), the UV-spectrum of the metabolite eluting after 6.26 min and the mass spectrum (negative ionization mode)

Table 3.2 Major metabolites formed by *Rhodococcus* ATCC 12674 (pDFDR) during transformation of dibenzofuran and related substrates

Substrate	Identified or possible products
Dibenzofuran	2,2′,3-Trihydroxybiphenyl
3-Chlorodibenzofuran	4-Chloro-2,2′,3-trihydroxybiphenyl
Dibenzo-*p*-dioxin	2,2′,3-trihydroxybiphenyl ether and putative 2,3-dihydrodiol dibenzo-*p*-dioxin
Carbazole	Unidentified single metabolite
2-Chlorodibenzofuran	No transformation

3.1.12 PCR amplification and detection of a second angular dioxygenase in *Rhodococcus* sp. strain HA01

The above cloned and characterized DfdA dibenzofuran dioxygenase showed a substrate spectrum and regioselectivity, which does not explain the observed transformation by the wild-type HA01. In contrast to HA01, recombinant DfdA did not transform 2CDBF at a measurable rate, and whereas DD was transformed mainly by angular dioxygenation in HA01, both lateral and angular dioxygenation were observed with recombinant DfdA dioxygenase. This indicated the presence of at least a second angular dioxygenase in HA01, which transforms dioxin predominantly by angular dioxygenation. To localize such second angular dioxygenase in HA01, new degenerated primers dxn1F and dxn2R (Table 2.4) were designed based on a multiple sequence alignment of the α-subunits of biphenyl and biarylether dioxygenases (2.10.2.2.1). As shown in Fig. 3.24 application of the primers to genomic DNA of *S. wittichii* RW1 successfully amplified the expected 700 bp fragment. Sequencing verified that the obtained fragment encoded the respective part of the α-subunit of dioxin dioxygenase. PCR reactions using the same primers with genomic DNA of *Rhodococcus* sp. strain HA01 (2.10.2.2.2) also amplified an

approximately 700 bp fragment (Fig. 3.24). The obtained nucleotide sequence of this fragment showed high similarities (98%) to the *dbfA* gene encoding the α-subunit of dibenzofuran dioxygenase of *Terrabacter* sp. strain DBF63 (AB054975) (Kasuga *et al.*, 2001). Primers sets designed from the angular dioxygenase encoding gene region from *Terrabacter* sp. strain DBF63 (dbfF2170/dbfR3600, dbfF3200/dbfR4600, F4275/R5400, dbfF4440/dbfR5900, and F5183/R6880ed01,2 were used for amplification of the complete angular dioxygenase encoding gene region from *Rhodococcus* sp. strain HA01.

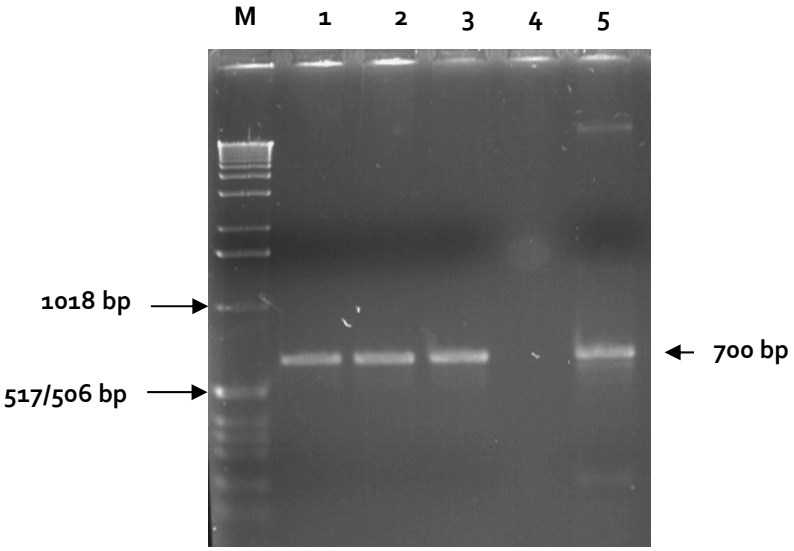

Fig. 3.24. Agarose gel electrophoresis of PCR products amplified with primers of dxn1F and dxn2R from genomic DNA of *Rhodococcus* sp. strain HA01 (lane 1 - 3) or *S.wittichii* RW1 (lane 5). Lane 4, negative control without template; M, molecular weight marker X (Roche). Arrows indicate the position of the approx. 700-bp fragment.

Sequence analysis revealed the presence of five open reading frames (ORF 1-5) on the obtained 4862bp fragment. The deduced amino acid sequence of a length of 443 amino acids of the protein encoded by ORF1, termed *dbfA1* thereafter, showed high homology (98.6% identity) with the α-subunit DbfA1 of the angular dibenzofuran dioxygenase from *Terrabacter* sp. strain DBF63 (Accession No.BAC75993). Similar identities were observed with the α-subunit of dibenzofuran dioxygenases of *Rhodococcus* sp. strain YK2 (Accession No. BAC00802, 98.9% identity) and *Rhodococcus* sp. strain DFA3 (Accession No. BAD51811, 98.3% identity), however, both last mentioned sequences were incomplete and do not cover the whole α-subunits. Interestingly, all three previously reported sequences were identical in the 213 amino acid stretch covered by all sequences, whereas the HA01 derived sequence differed in this stretch by 3 amino acids. The deduced amino acid sequence of the 167 amino acids protein encoded by ORF2, termed *dbfA2* thereafter, located downstream of *dbfA1*, showed 98.2% of identity with either the β-subunit of the dibenzofuran dioxygenase (DbfA2) from *Terrabacter* sp. strain DBF63 (Accession No. BAC75994) or from *Rhodococcus* sp. strain YK2 (Accession No. BAC00803).

Likewise, the deduced amino acid sequence of the 319 amino acids protein encoded by ORF3 located downstream of ORF2 showed high homology (98.4% identity) to putative *meta*-cleavage compound

hydrolases encoded downstream of the α- and β-subunits of dibenzofuran dioxygenase of *Rhodococcus* sp. strain YK2 and *Terrabacter* sp. strain DBF63. A similar protein was also reported to be encoded by the genome of *Rhodococcus rhodochrous* K37, a strain described to have a multiplicity of extradiol dioxygenase (97.6% identity in a stretch of 126 amino acid analyzed) (Taguchi *et al.*, 2004).

Like in *Rhodococcus* sp. strain YK2, *Terrabacter* sp. strain DBF63, and *Rhodococcus rhodochrous* K37, the gene encoding a putative *meta*-cleavage compound hydrolase (ORF3) was followed in HA01 by a gene encoding a putative extradiol dioxygenase (ORF4). The respective protein, however, does not belong to the type I (vicinal oxygen chelate superfamily) extradiol dioxygenases typically involved in the degradation of biphenyl and related compounds, but to the type II extradiol dioxygenases which include enzymes such as protocatechuate 4,5-dioxygenase (LigAB) from *Pseudomonas paucimobilis* (Noda *et al.*, 1990) which has two different types of subunits. The deduced amino acid sequence of the protein encoded by ORF4 showed 99.3% identity with the respective proteins from *Rhodococcus* sp. strain YK2 and *Rhodococcus rhodochrous* K37, but only 94.3% of identity with that from *Terrabacter* sp. strain DBF63. The relatively low homology was mainly due to an 8 amino acid gap at the N-terminus of the DBF63 protein.

The deduced amino acid sequence of the 81 amino acid protein encoded by ORF5 located downstream of ORF4 showed high homology (98.7 % identity) to the ferredoxin proteins of *Rhodococcus* sp. strain YK2 and *Rhodococcus rhodochrous* K37 but relatively low homology (90%) with the ferredoxin gene of *Rhodococcus* sp. DFA3.

3.1.13 Expression of *dbfA* genes in *Rhodococcus* sp. strain HA01

To identify if, like the *dfd* genes, also the *dbf* genes are expressed in response to DBF, RT-PCR experiments were performed with total RNA extracted from *Rhodococcus* sp. strain HA01 growing on DBF and fructose. RT-PCR amplification products of the expected 700 bp size were observed from RNA extracted from the culture grown on DBF (Fig. 3.25 A, lane 1 - 13), using FDBFRNA and RDBFRNA primers annealing in the *dbfA1* gene, whereas no product was detected with RNA extracted from the culture grown on fructose (Fig. 3.25 B, lane 1 - 13).

In addition, no amplification products were observed in controls devoid of reverse transcriptase (Fig. 3.25 A and B lane 14-15) or template cDNA (Fig. 3.25 A and B lane 16). Sequencing of he approximately 700 bp product confirmed that it was identical to the corresponding *dbfA1* gene fragment from genomic DNA of *Rhodococcus* sp. strain HA01. These results showed that also the DbfA dioxygenase from *Rhodococcus* sp. strain HA01 is specifically induced in the presence of DBF and, thus, at least two obviously angular dioxygenases are simultaneously expressed when HA01 is confronted with DBF.

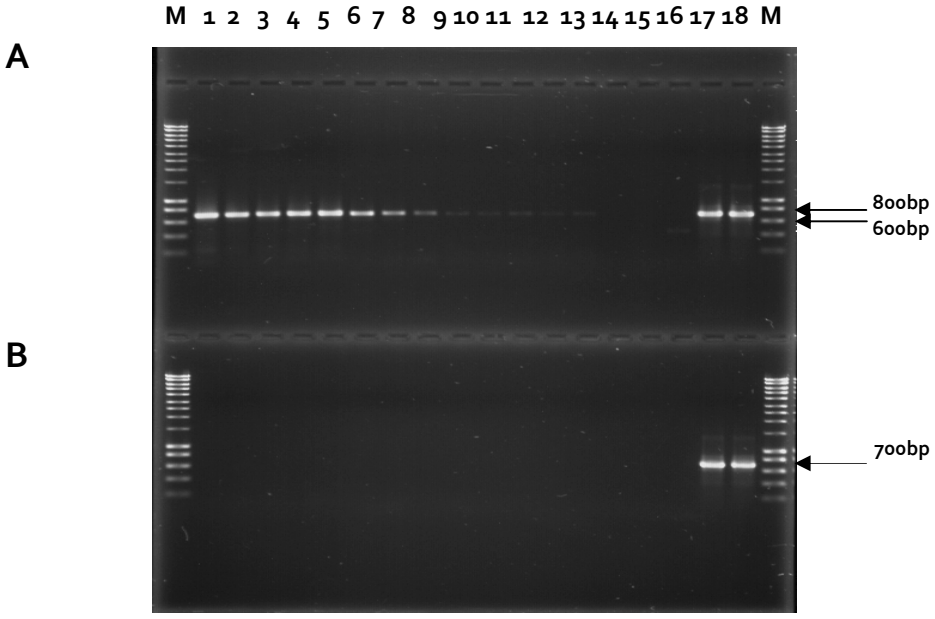

Fig. 3.25. RT-PCR amplification of *dbfA* mRNA from *Rhodococcus sp*. strain HA01 grown on DBF (Panel A) or fructose (Panel B). M, molecular weight marker Hyperladder 1(Bioline). cDNA generated from template RNA was serially diluted (3.2-fold) with nuclease-free water and 1 µl of each dilution was subjected to amplification by PCR (lanes 1-13). Negative controls included RT and PCR reactions devoid of reverse transcriptase (lanes 14-15) or template cDNA (lane 16), respectively. PCR reactions containing 1 ng of genomic DNA as a template were used as positive control (lanes 17-18)

3.1.14 Heterologous expression of DbfA dioxygenase from *Rhodococcus* sp strain HA01

3.1.14.1 Expression in *E. coli* using pUC119

As *dbfA1A2* from *Terrabacter* sp. strain DBF63 was cloned using pUC119 (pDF32) in *E.coli* JM109 and some activity against DBF was observed using this host (Kasuga *et al.*, 2001), a 3kb DNA fragment (Fig. 3.26) containing the gene region comprising the *dbfA1A2*genes encoding the α-and β subunit of DbfA dioxygenase from *Rhodococcus* sp. strain HA01 was amplified using the forward primer FHind3DBF63 and reverse primer RECODBF63 which include artificial restriction sites for *HindIII* and *EcoR1*, respectively. After confirmation of the sequence, the fragment was cloned into *HindIII* and *EcoR1* sites of pUC119 giving (pDBFA12a) and transformed by heat shock into *E. coli* JM109. Resting cells of *E.coli* (pDBFA12a) were prepared after growth in the presence of 0.5 mM IPTG. When those cells (OD_{600nm} = 10) were incubated in the presence of 0.3 mM DBF, 3CDBF, 2CDBF, or DD, a faint activity was observed only against DBF and after 18 hours of incubation approximately 2µM of THB were produced, as evidenced by HPLC analysis and comparison with an authentic standard. Activity against all other substrates was below the detection limit.

3.1.14.2 Expression in *E. coli* via pRSG43

A 3 kb DNA fragment (Fig. 3.26) containing the gene region comprising the *dbfA1A2* genes encoding the α-and β subunits of DbfA dioxygenase was amplified by PCR from *Rhodococcus* sp. strain HA01 genomic DNA using the forward primer FXBA1DBF63 and the reverse primer RECODBF63 which include artificial

restriction sites for *Xba*1 and *EcoR1* respectively. After confirmation of the sequence, the fragment was digested by *Xba*1 and *EcoR1* restriction enzymes, cloned into the *Xba*1 and *EcoR1* sites of the *Rhodococcus* – *E. coli* shuttle vector pRSG43 giving pDBFA12 and transformed by heat shock into *E. coli* JM109. At the same time, as a negative control, *E.coli* JM109 was transformed with the shuttle vector pRSG43 without insert. Presence of the 3 kb insert in pDBFA1A2 was confirmed by colony PCR using two different primer sets FDBFRNA/RDBFRNA and F3200/R4600 annealing in the α-subunit and β subunit genes respectively. PCR products of the expected length were obtained from clones containing pDBFA12, whereas PCR products were absent when clones containing pRSG43 were analyzed.

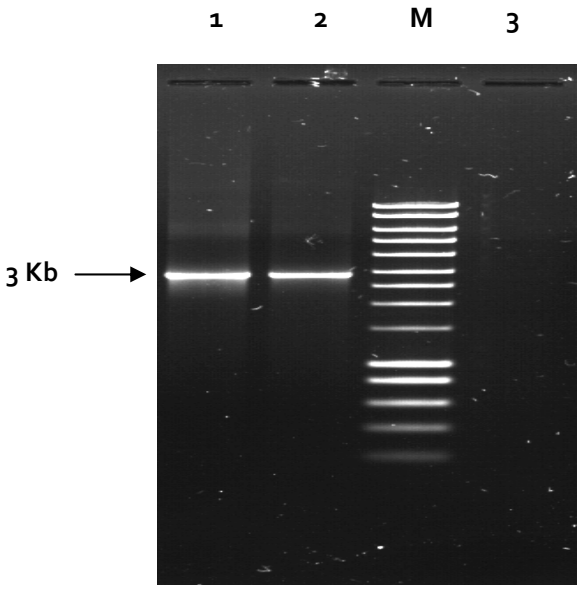

Fig. 3.26. Agarose gel electrophoresis of PCR products amplified with primers FHind3DBF63 and RECODBF63 from genomic DNA of *Rhodococcus* sp. strain HA01 (lane 1 and 2), Lane 3, negative control without template; M, molecular weight marker Hyperladder I (Bioline). Arrows indicate the position of the approx. 3 kb fragment

3.1.14.2.1 Analysis of dbfA1A2 expression by SDS-PAGE

To analyze whether the dbfA1A2 genes on pDBFA12 were expressed and translated to polypeptides with the predicted sizes in E.coli JM109, extracts of cells harboring pRSG43 and those of cells harboring pDBFA12 were compared by SDS-PAGE. As shown in Fig. 3.27, a prominent band of a molecular mass of approximately 49 kDa was observed in cell extracts of *E. coli* JM109 (pDBFA12), which was absent in cell extracts of *E. coli* JM109 (pRSG43). This is in agreement with the expected molecular mass of the dbfA1 gene product (49.5kDa).

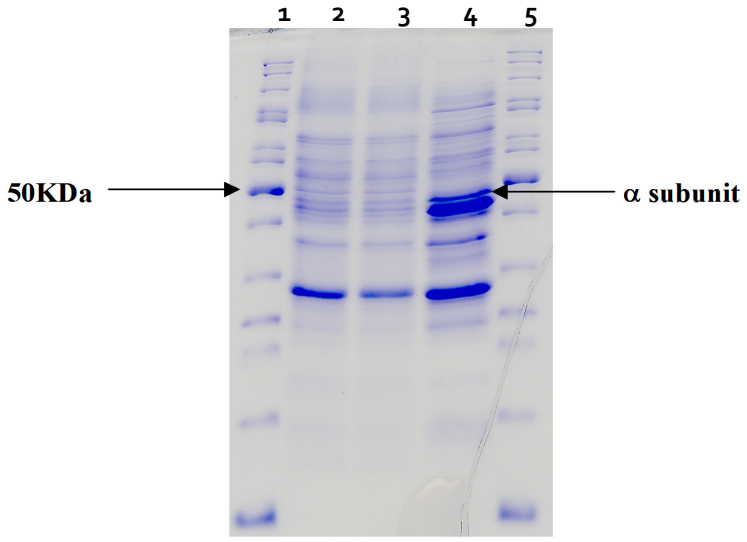

Fig. 3.27. SDS-PAGE analysis of cell extracts of *E.coli* JM109 (lane 2), *E.coli* JM109 (pRSG43)(lane 3), and of *E.coli* JM109 (pDBF12) induced with IPTG (lane 4). Lane 1 and 5, Page Ruler unstained protein ladder (MBI Fermentas)

3.1.14.2.2 Transformation of DBF, DD, 2CDBF, 3CDBF, and carbazole by E.coli JM109 (pDBFA12)

To analyze if an active dioxygenase was present in cells of *E.coli* JM109 (pDBFA12), resting cells of a culture pregrown in the presence of 0.5 mM IPTG were prepared (OD_{600nm} = 10) and incubated with 0.3 mM DBF, DD, 2CDBF, 3CDBF, or carbazole for 3 hours at 30°C. Only single products were observed during transformation of DBF and DD. HPLC analysis revealed these metabolites to cochromatograph with authentic THB and THBE standards and to reveal UV absorption spectra identical to the respective standards, evidencing DBF and DD to be dioxygenated exclusively at the angular positions. 2CDBF was transformed into a single product, which was neither identical to either 5-chlorosalicylate nor 2-chloro-3,4-dihydro-3,4-dihydroxydibenzofuran formed by *Rhodococcus* sp. strain HA01. It can thus be proposed that the observed metabolite has the 5′-chloro-2,2′,3-trihydroxybiphenyl structure, which in HA01 is further transformed into 5-chlorosalicylate. No activity was observed with 3CDBF, even after extended incubation for 12 hours.

Carbazole was transformed into two products, exhibiting retention volumes of 3.4 min and 5.9 min, respectively when an eluent containing 56 % methanol was used. The metabolite eluting at 3.4 min showed a retention behavior and UV absorption spectrum identical to the single metabolite produced from carbazole by *Rhodococcus* sp. ATCC12674 (pDFDR) expressing DfdA dioxygenase.

3.1.14.3 Expression in *Rhodococcus* sp. ATCC 12674 using the *Rhodococcus* - *E. coli* shuttle vector pRSG43

To evaluate if higher activities could be obtained by expression in *Rhodococcus*, pDBFA12 was introduced into *Rhodococcus* sp. ATCC 12674 by electroporation (2.8.8.3). However, resting cells of *Rhodococcus* sp. ATCC 12674 (pDBFA12) of an OD_{600nm} of up to 20 did not show any detectable transformation of neither DBF, 3CDBF, 2CDBF nor DD or any detectable product formation in incubations of up to 24 hours.

3.2 Analysis of *Sphingomonas wittichii* RW1 and its mutants M2 and M10

Besides the initial dioxygenation, which, for successful mineralization, has to occur at the angular positions, also the ring-cleavage of dihydroxylated intermediates can be regarded as a crucial step in the mineralization of biarylethers. Previous analysis had indicated, that DbfB extradiol dioxygenase, which was assumed to be the crucial enzyme expressed during growth on DBF was rapidly inactivated during the transformation of THBE (Happe *et al.*, 1993), the metabolite of DD degradation, indicating that beside DbfB, other extradiol dioxygenases are of crucial importance for DD mineralization by this strain. In fact, two additional genes *edo2* and *edo3* encoding extradiol dioxygenases had also previously been identified in the genome of strain RW1 (Armengaud *et al.*, 1998; D'Enza, 2002).

3.2.1 Extradiol dioxygenase activity in *Sphingomonas wittichii* RW1 and its mutants M2 and M10

To evaluate the importance of DbfB for the degradation of DBF and DD by RW1, two dbfB knock out mutants of RW1 termed M2 and M10 were analyzed. These mutants had previously been obtained by exchange of the wildtype *dbfB* gene in the chromosome of RW1 against a *dbfB* gene interrupted at the single NotI restriction site by the kanamycin resistance gene from pUTXylkE (de Lorenzo *et al.*, 1990), through homologous recombination (Happe, B., unpublished).

Despite the inactivation of the *dbfB* gene, both mutants were still capable to grow on DBF and DD, indicating the recruitment of another extradiol dioxygenase for mineralization.

To analyze for the differences in extradiol dioxygenase activities, activities of wild type RW1 and mutants against different substrates were evaluated in differently pregrown cells using cell extracts as well as resting cells. As previously described (Wittich *et al.*, 1992) high extradiol dioxygenase activity with DHB (up to more than 4000 U/mg, see Fig. 3.28) was observed in cell extracts of RW1 cells pregrown on DD, DBF, or salicylate, and lower activity in extracts of cells pregrown on acetate. In contrast, when cell extracts of the mutants M2 or M10 were prepared and analyzed under identical conditions, activity with DHB was neither observed in extracts of DD, DBF nor of salicylate grown cells.

To analyze if the failure to detect 2,3-dihydroxybiphenyl 1,2-dioxygenase (DHB12O) activity was due to problems in constituting an active enzyme in cell extracts, activities of RW1 were determined with DD, DBF and salicylate grown resting cells. A rapid coloration due to *meta*-cleavage of DHB was observed in all cases. Quantification of transformation rates by HPLC revealed, like in cell extracts, highest DHB12O activities in DBF grown cells, and slightly lower activities in DD or salicylate grown cells (Fig. 3.29). However, all these cells also exhibited significant activities with THBE, whereas HPLC analysis revealed no significant THBE transformation by any cell extract. High activities with catechol in DD grown cells are obviously due to induction of a catechol 1,2-dioxygenase activity, which is reported to be absent in salicylate or DBF grown cells (Wittich *et al.*, 1992). Importantly, whereas cell extracts of the *dbfB* knock out mutants M2 and M10 showed neither a transformation of DHB nor of THBE, specifically DD and DBF

grown resting cells showed a rapid transformation of DHB and a significant turnover rate with THBE, in case of DHB transformation concomitantly with a yellow coloration due to accumulation of the *meta*-cleavage product (Fig. 3.30 and 3.31). Overall, these results indicate the presence of at least one enzyme in M2 and M10, which is active against THBE and DHB, but can not be detected in cell extracts using the standard enzyme test for DHB12O. Moreover, it can be postulated that this activity is responsible for effective THBE transformation also in RW1. Extradiol dioxygenases are known to be subject to inactivation in the presence of oxygen due to the oxidation of active site Fe^{2+} to Fe^{3+} (Vaillancourt *et al.*, 1998; Vaillancourt *et al.*, 2002; Vaillancourt *et al.*, 2006), and significant differences in susceptibility to oxidative inactivation had been observed. As one hypothesis, we assumed the THBE transforming activity to be specifically susceptible to oxidation. Attempts to stabilize this activity by preparation of cell extracts under oxygen limiting conditions, however, failed and DHB12O activity could neither be observed in cell extract of DD, DBF, nor of salicylate grown cells of strains M2 or M10 using the spectrophotometric test. Extradiol dioxygenase are usually Fe^{2+}-dependent enzymes, however, extradiol dioxygenase dependent on Mn^{2+} as cofactor have also been described (Hatta *et al.*, 2003; Miyazawa *et al.*, 2004; Que & Reynolds, 2000; Reynolds *et al.*, 2003; Sakurai *et al.*, 1983; Whiting *et al.*, 1996). To test if the absence of activity was due to the absence of an essential bivalent ion, cell extracts were prepared under oxygen limiting conditions and supplemented with 2mM Fe^{2+}, Cu^{2+}, Mn^{2+} or Mg^{2+}. However, DHB12O activity was observed neither in cell extracts of DBF nor DD grown cells of strains M2 or M10, even when the mentioned metals were separately included also in the enzyme test.

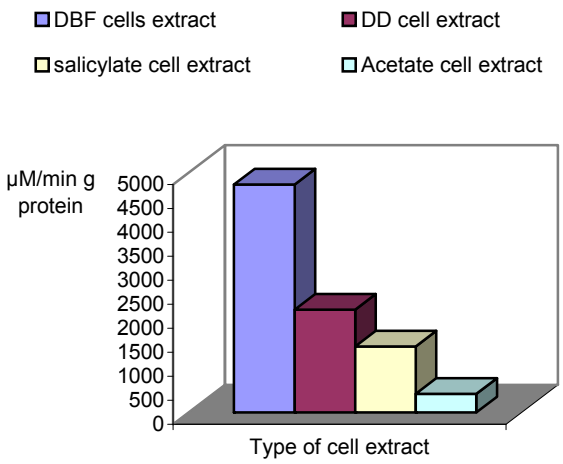

Fig. 3.28. 2,3-Dihydroxybiphenyl 1,2-dioxygenase activity in cell extracts of *Sphingomonas wittichii* RW1 after growth with different substrates. Activities were determined spectrophotometrically.

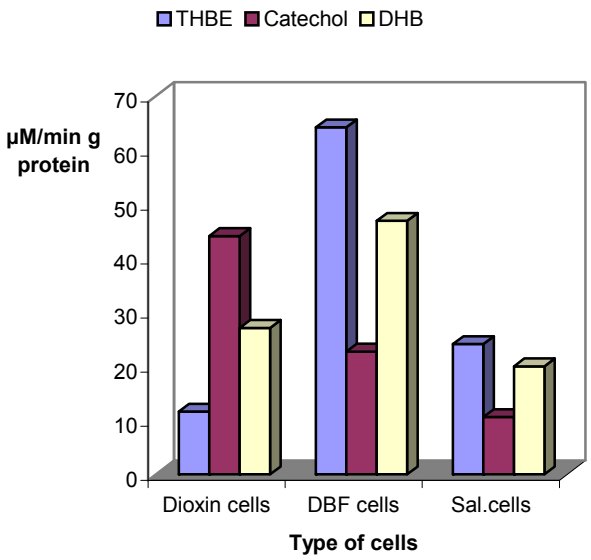

Fig. 3.29. Transformation of THBE, catechol, and DHB by resting cells of *Sphingomonas wittichii* RW1 pregrown on DD, DBF or salicylate, respectively. Transformation was quantified by determination of substrate depletion by HPLC.

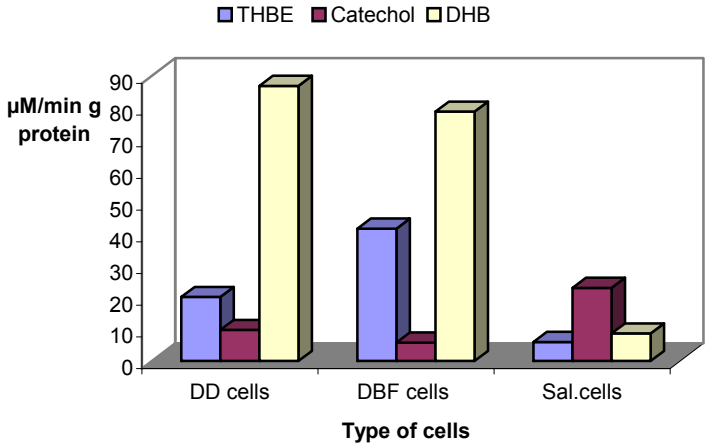

Fig. 3.30. Transformation of THBE, catechol, and DHB by resting cells of strain M2 pregrown on DD, DBF or salicylate, respectively. Transformation was quantified by determination of substrate depletion by HPLC.

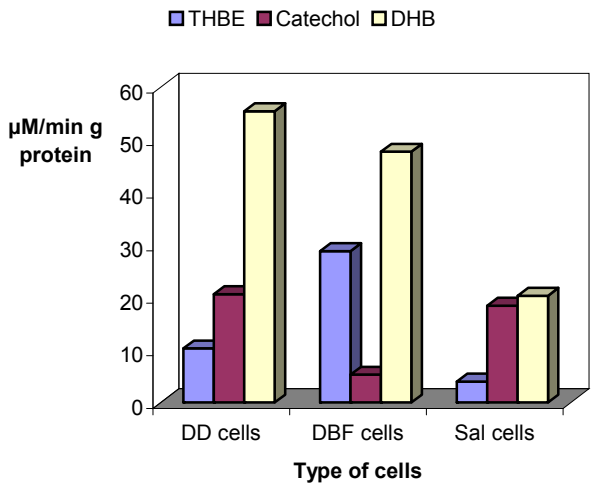

Fig. 3.31. Transformation of THBE, catechol, and DHB by the resting cells of strain M10 pregrown on DD, DBF or salicylate, respectively. Transformation was quantified by determination of substrate depletion by HPLC.

3.2.2 Characterization of novel extradiol dioxygenases from *Sphingomonas wittichii* RW1

As biochemical strategies to characterize in cell extract the extradiol dioxygenase activity active against THBE failed, genetic approaches were used. A useful approach is the cloning of whole genome fragments, termed a genomic library in expression vectors, followed by screening of the library for the activity of interest.

3.2.2.1 Identification of extradiol dioxygenase encoding genes by the use of phage libraries

To test if the approach using genomic libraries is useful to localize genes encoding extradiol dioxygenases, genomic DNA was isolated from DBF grown cells of RW1. For preparing the genomic library in the λ-ZAP Express vector, fragment sizes of approximately 5 kb are necessary. To obtain fragment sizes of 5 - 10 kb, partial digestions of genomic DNA (0.85 µg/µl) were performed with different concentrations of *Sau*3A (0.0015 – 4 U/µl for 1 h) to evaluate optimal partial restriction. Finally, 20 µg of genomic DNA was digested with 4 U/µl of *Sau*3A (2.10.10.1). The digested DNA was size-fractioned by ultracentrifugation in a 10 - 40% sucrose gradient (2.10.10.2) and fractions containing fragments of the required size were used for ligation and packaging (2.10.10.3).

The genomic library constructed in λ-ZAP Express vector was shown to comprise 1.5 x 10^6 pfu/µl. Taking into account the recently determined genome size of *Sphingomonas wittichii* RW1 as 5.915 Mb (http://genome.jgi-psf.org/draft_microbes/sphwi/sphwi.home.html), and assuming a mean insert size of roughly 5 kb, it can be calculated that approximately 11,000 plaques will correspond to a 1- fold genome equivalent. As described above, three different genes encoding extradiol dioxygenases, which all, under standard assay conditions were found to be relatively stable, had been observed in RW1 (*dbfB*, *edo2*, and *edo3*). Assuming each extradiol dioxygenase to be encoded once on the genome, 3 out of 11,000 plaques should show extradiol dioxygenase activity. However, out of 11,000 plaques analyzed, none of them

showed yellow coloration after spraying with DHB indicating the absence of active extradiol dioxygenase in all of them.

3.2.2.2 Identification of extradiol dioxygenase encoding genes by the use of phagemid libraries

As we assumed the absence of extradiol dioxygenase activity in any of the plaques analyzed to be due to exposure of the extradiol dioxygenase in thin layer to oxygen and thus inactivation, the phage library was converted into a phagemid library (2.10.10.6). A total of 11,000 *E .coli* XLOLR colonies that were supposed to contain vector with the cloned DNA inserts were sprayed with DHB, and 24 colonies showed a yellow coloration indicating these colonies to contain a phagemid harboring a gene encoding an extradiol dioxygenase. Specific primers set for amplifying *dbfB* (PdfBF1/PdfBR1), *edo2* (Edo2F1/Edo2R1), and *edo3* (Edo3FHH/Edo3RHH) were used for identifying phagemids that contain known inserts. All 24 positive phagemids showed amplification with the *edo2* primer set and no amplification was obtained with either the *dbfB* or *edo3* primer set. Sequencing of the approximately 690 bp products confirmed that they were identical to the corresponding *edo2* gene fragment of RW1.

3.2.2.3 Identification of extradiol dioxygenase encoding genes by the use of fosmid libraries

Recently, a fosmid library had successfully been prepared to analyze the diversity of extradiol dioxygenases from a contaminated site, and it could be shown, that such genes could easily be expressed from fosmids in *E. coli* (Suenaga. *et al.*, 2007). As each fosmid clone contains an approx. 40 kb insert, the whole genome of a single organism can be covered by a few hundreds of fosmid clones (Camara, 2007). The constructed *Sphingomonas wittichii* RW1 fosmid library which comprised a total of approximately 3000 recombinant clones was screened for extradiol dioxygenase activity by spraying colonies with DHB. A total of 95 clones showed such activity. To analyze which of these activities is due to already known extradiol dioxygenases, DbfB, Edo2, or Edo3, the above described primer sets (PdfBF2/PdfBR2, Edo2F2/Edo2R2, Edo3FHH/Edo3RHH) were used for screening by PCR. Whereas 66 fosmids obviously contained the *dbfB* gene, and 25 fosmids contained the *edo2* gene, none of the fosmids contained the *edo3* gene (Fig. 3.32). Four clones, (3, 7, 8, and 9) did not yield amplification products with any of above mentioned primers and thus can be supposed to contain a previously uncharacterized extradiol dioxygenase.

Four clones, (3, 7, 8, and 9) did not yield amplification products with any of above mentioned primers and thus can be supposed to contain a previously uncharacterized extradiol dioxygenase. These four clones as well as one clone expressing DbfB (as positive control) were grown and tested for transformation of DHB in cell extracts. All five extracts showed extradiol dioxygenase activity and thus do not express the supposedly "unstable" extradiol dioxygenase assumed to be important for DD degradation.

To analyze if a THBE transforming activity is expressed by clones of the fosmid library, a total of 384 randomly selected clones were tested for capability to transform THBE (supplied at a concentration of 50 µM). *Sphingomonas wittichii* RW1 was included in this screening as positive control. However, HPLC

analysis did not give evidence for transformation by any clone, whereas THBE was quantitatively transformed by the positive control.

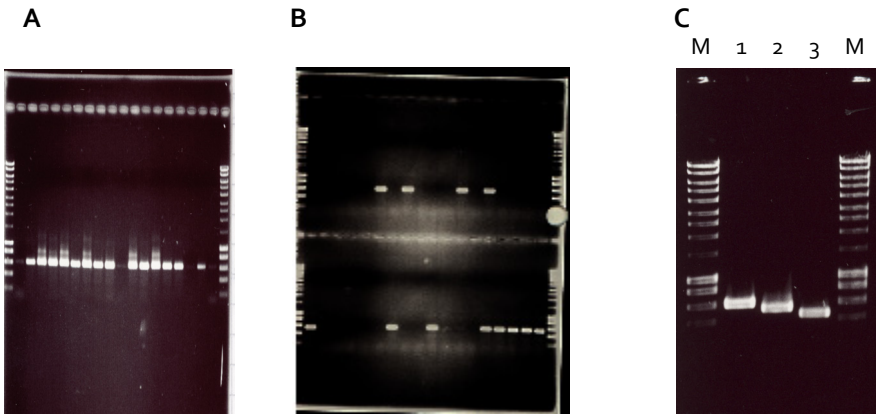

Fig. 3.32. Screening of fosmid clones by PCR using the *dbfB* specific primer set (PdbfBF2/PdbfBR2) (A), and the *edo2* specific primer set (Edo2F2/EdoR2) (B). In panel A, 14 out of 18 fosmids showed an amplification product whereas in panel B, 12 out of 36 fosmids showed an amplification product. Panel C shows the positive control reactions using *dbfB* (PdbfBF2/PdbfBR2, lane 1), *edo2* (Edo2F2/EdoR2, lane 2), and *edo3* (Edo3FHH/Edo3RHH lane 3) specific primers on RW1 genomic DNA as template, M, molecular weight marker Hyperladder 1 (Bioline)

3.2.2.4 Identification of additional genes belonging to the extradiol dioxygenase type I family in the genome of strain RW1

As there are indications pointing out for the presence of functional extradiol dioxygenase genes additional to the ones already identified on strain RW1, we used a genomic approach in order to identify them. During the course of this study, different sequenced fragments of the genome sequencing project of strain RW1 had been released in sequence databases. The unfinished draft comprised 29 contigs summing up for a total genome size of approximately ~5.915 Mb. The contigs could be found under GenBank accession number NZ_AAVK00000000 (November 2006). Recently the contigs were assembled and the genome sequence, as well as the sequence of two plasmids, are available under CP000699 to CP000701 (May 2007).

The genome sequence contigs were independently compared for similarities by using the tool bl2seq and selecting NCBI BlastN program against a database of concatenated coding DNA sequences (CDS) of the Extradiol Dioxygenases type I, which comprises 197 gene members that represent the main evolutionary branches in that family (H. Junca, personal communication). It was possible to detect four sequences or putative loci on the RW1 genome, producing significant similarity values with some of the extradiol dioxygenase sequences compared. The precise location of the positive sequences on the contigs were defined, and the putative open reading frames (ORFs) that would theoretically encode an extradiol dioxygenase protein were extracted from this genome sequence database.

Among the sequences producing significant similarities, all generically annotated as "Glyoxalase/bleomycin resistance" (the superfamily where extradiol dioxygenases type I belongs to), one

was localized close to a FAD monooxygenase-like gene. The encoded protein has 62% identity with 2-hydroxybiphenyl-3-monooxygenase from *Pseudomonas azelaica* (Jaspers *et al.*, 2000), indicating a possible involvement of the mentioned extradiol dioxygenase gene in aromatic biodegradation. The conceptual translation of the corresponding extradiol dioxygenase ORF (termed *edo4* thereafter, and designated Swit3046 in the *Sphingomonas wittichii* RW1 genome project, CP000699) shows a protein with conserved motifs of 2,3-dihydroxybiphenyl dioxygenases. However the maximum similarity with all extradiol dioxygenases reported so far is only 58 % identity in the amino acid sequence with the 2,3-dihydroxybiphenyl-1,2-dioxygenase (HbpC) from *Pseudomonas azelaica* HBP1(Jaspers *et al.*, 2000) and 51% with the 2,3-dihydroxybiphenyl 1,2-dioxygenase (PcbC) of *Pseudomonas* sp. strain DJ-12 (Kim *et al.*, 1996), thus representing a new evolutionary branch of the extradiol dioxygenase family, similarly as the situation previously encountered in the Rieske non heme dioxygenases found in RW1 (Armengaud *et al.*, 1998). The ORF termed *edo5* (Swit3298, CP000699) putatively encoding a protein with conserved motifs of 2,3-dihydroxybiphenyl 1,2-dioxygenases exhibited highest similarity (44% of identity) with 2,3-dihydroxybiphenyl 1,2-dioxygenases from *Pseudomonas* sp. strain SY5 (Accession no. AAF04139) and *Ralstonia eutropha* H16 (Accession no. CAJ95451). Edo 6 (Swit1848, CP000699) shows 37% identity with 2,3-dihydroxybiphenyl 1,2-dioxygenase from *Rhodococcus erythropolis* TA421 (Kosono *et al.*, 1997) and Edo7 (Swit4182, CP000699) exhibits 47% identity with 2,3-dihydroxybiphenyl 1,2-dioxygenase from *Rhodococcus* sp. M5 (Wang *et al.*, 1995), and 2,3-dihydroxybiphenyl 1,2-dioxygenase I from *Rhodococcus globerulus* P6 (Asturias *et al.*, 1994). The protein encoded by an additional ORF termed edo8 (Swit1756, CP000699) shows 47% identity with a putative 2,3-dihydroxy-p-cumate-3,4-dioxygenase (CmtC) from *Burkholderia xenovorans* LB400 (Accession no. ABE29434) and 46% identity with 2,3-dihydroxy-p-cumate 3,4-dioxygenase of *Pseudomonas putida* F1 (Eaton, 1996) indicative for the possible involvement of Edo8 in p-cumate degradation. Five primer sets (FedoN16RW1/RedoN16RW1, 5fedorw1/5Redorw1, 6fedorw1/6Redorw1, 7fedorw1/7Redorw1, and edo8fRW1/edo8RW1) were designed for the amplification of 992bp, 1010bp, 1071bp, and 1004 bp fragments, respectively, from genomic DNA of RW1 comprising the complete *edo4, edo5, edo6*, and *edo7* ORFs, respectively, but also to evaluate if the expression of extradiol dioxygenase activities in fosmids 3, 7, 8, and 9 is due to the presence of any of the *edo4, edo5, edo6*, or *edo7* ORFs on the respective fosmid. PCR-based screening indicated *edo5* to be located on all 4 mentioned fosmids, suggesting that *edo5* encodes a functional 2,3-dihydroxybiphenyl 1,2-dioxygenase.

To analyze if the *edo4, edo5, edo6* and *edo7* ORFs encode functional extradiol dioxygenases, amplified fragments described above were separately cloned into pGEM®-T Easy Vector and transformed by heat shock in *E.coli* JM109. Screening of colonies carrying *edo4, edo5, edo6* or *edo7* inserts by spraying with DHB showed that all exhibited extradiol dioxygenase activity, and thus that all putative ORFs encode functional EDO proteins. *E. coli* JM109 cells carrying the pGEM®-T Easy Vector with any of the four inserts were grown and tested for transformation of DHB by assays with resting cells assays and cell extracts. All four cell types showed extradiol dioxygenase activity in resting cells but also in cell extracts

and thus do not express the supposedly "unstable" extradiol dioxygenase assumed during the analysis of the mutants M2 and M10.

3.2.2.5 Comparison of kinetic properties of previously identified extradiol dioxygenases (DbfB, Edo2 and Edo3) from *Sphingomonas wittichii* RW1

Out of the extradiol dioxygenases from *Sphingomonas wittichii* RW1, only DbfB has been described in some more detail (Happe *et al.*, 1993). However, despite the observation of a rapid inactivation of DbfB during transformation of THBE, no detailed analysis of this inactivation has been performed. The same holds for Edo2 and Edo3 dioxygenases.

The activity of the different extradiol dioxygenases, in cell free extracts of *E. coli* BL21 (DE3)[LysS](pT7-5RW), *E. coli* BL21 (DE3)[LysS](pT7-22), or *E. coli* BL21 (DE3)[LysS] (pT7W4) expressing DbfB, Edo2 or Edo3, respectively, was always 9.000 ± 2000 U/g protein for DbfB, and significantly lower in the case of Edo2 (2700 ± 700 U/g protein) and Edo 3 (2100 ± 800 U/g protein).

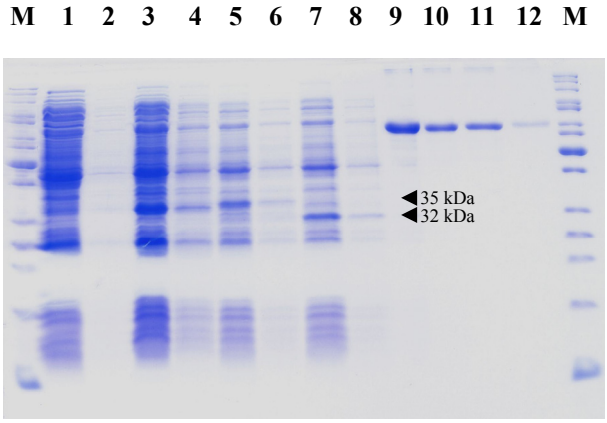

Fig. 3.33. SDS PAGE of cell extracts of *E. coli* BL21 (DE3)[LysS] (lane 1 and 2, 28 µg and 7 µg of protein, respectively) *E. coli* BL21 (DE3)[LysS] (pT7-5RW)(lane 3 and 4, 28 µg and 7 µg of protein, respectively), *E. coli* BL21 (DE3)[LysS] (pT7-22 (lane 5 and 6, 15 µg and 4 µg of protein, respectively), or *E. coli* BL21 (DE3)[LysS] (pT7W4) (lane 7 and 8, 14 µg and 3.5 µg of protein, respectively). M, Page Ruler unstained protein ladder (MBI Fermentas). Lanes 9 - 12, show electrophoresis of 2 µg, 1.5 µg, 1.0 µg and 0.5 µg, respectively, of a BSA standard

Basic kinetic data (see Table 3.3) had previously been determined mainly in cell extracts by d'Enza (D'Enza, 2002), however, turnover numbers were not calculated. Comparison of the protein composition of cell extracts of *E. coli* BL21 (DE3)[LysS] expressing DbfB, Edo2 or Edo3 indicated the presence of additional protein bands of molecular masses similar to those predicted for DbfB (32.3 kDa), Edo2 (35.2 kDa) or Edo3 (32.3), respectively (Fig. 3.33).

Table 3.3: Catalytic properties of different 2,3-dihydroxybiphenyl 1,2-dioxygenases originating from *Sphingomonas wittichii* RW1 (D'Enza, 2002). The kinetic parameters were determined using cell extracts of *E. coli* BL21(DE3)[LysS] expressing Edo2 and 3, respectively and compared to those described for DbfB by Happe et al. (Happe *et al.*, 1993). K_m values are expressed in µM for all substrates. Maximal turnover rates are expressed relative to those determined with 2,3-dihydroxybiphenyl as substrate. As turnover numbers were not calculated, the V_{max} /K_m values were compared relative to that for 2,3-dihydroxybiphenyl set as 100, as indication for the specificity constant k_{cat}/K_m.

Substrate	Edo 2			Edo 3			DbfB		
	K_m (µM)	V_{max} (%)	V_{max}/K_m (%)	K_m (µM)	V_{max} (%)	V_{max}/K_m (%)	K_m (µM)	V_{max} (%)	V_{max}/K_m (%)
2,3-dihydroxybiphenyl	**140±23**	**100**	**100**	**55 ± 5**	**100**	**100**	**8 ± 2**	**100**	**100**
catechol	**830±270**	**30**	**5**	**4000 ± 900**	**3**	**0.04**	**83000 ± 4000**	**42**	**0.004**
3-methylcatechol	**410 ± 80**	**35**	**12**	**10500 ± 1500**	**26**	**0.14**	**5300 ± 600**	**29**	**0.04**
2,2',3-trihydroxybiphenyl	**40 ± 15**	**28**	**98**	**30 ±5**	**90**	**165**	**11 ± 1**	**30**	**22**

As the quantity of extradiol dioxygenase in extracts of cells overexpressing them could be only very roughly determined, all three enzymes were subject to partial purification by anionic exchange chromatography. When a cell extract containing an amount of DbfB corresponding to an activity of 78.8 U (measured with 0.1 mM DHB) was separated, the activity (eluting at 0.28 ± 0.02 M NaCl) was completely (>90%) recovered. High recovery was also observed during partial purification of Edo3 (eluting at 0.35 ± 0.02 M NaCl). Of an applied amount of 4.54 U, 3.4 U could be recovered, which corresponds to a recovery of 75%. In contrast, Edo2, eluting at 0.32 ± 0.02 M NaCl, was rather unstable and only 30% of the applied activity was recovered. In the most enriched fractions, Sypro ruby quantification indicates DbfB to comprise 36.0 %, Edo2 to comprise 13.2% and Edo3 to comprise 67.1 % of the whole protein in the respective fractions (Fig. 3.34).

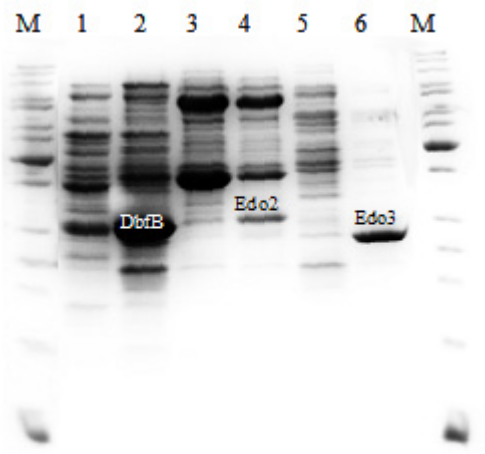

Fig. 3.34. SDS PAGE of protein fractions eluting at 0.28 M NaCl (lane 1 and 2), 0.35 M NaCl (lane 3 and 4) and 0.32 M NaCl (lane 5 and 6) during anionic exchange chromatography of *E. coli* BL21 (DE3)[LysS] (lane 1, 3 and 5), *E. coli* BL21(DE3)[LysS] (pT7-5RW) (lane 2), *E. coli* BL21(DE3)[LysS] (pT7-22) (lane 4), or *E. coli* BL21(DE3)[LysS] (pT7W4) (lane 6). 4 – 8 µg of protein were separated. M, Page Ruler unstained protein ladder (MBI Fermentas)

The specific activities (V_{max} values) in these fractions could be determined as 116,500 U/g protein, 9,300 U/g protein and 8,000 U/g protein, respectively, corresponding to activities of 323,700 U/g DbfB, 70,800 U/g Edo2, and 10,700 U/g Edo3, respectively. Assuming inactivation during purification to be due to oxidation of active site Fe^{2+}, and protein bands quantified by Sypro ruby staining to comprise both "active" and "inactive" enzyme species, it can be calculated that active DbfB, Edo2 and Edo3 have activities of 323,700 U/g (no inactivation during purification), 235,900 U/g (recovery of 30% of activity indicated 70% of the Edo2 protein to be inactive) and 14,200 U/g (recovery of 75% of activity indicated 25% of the Edo3 protein to be inactive), respectively, which, based on subunit molecular masses of 32.3 kDa, 35.2 kDa and 32.3 kDa, respectively would translate to k_{cat} values of 167 s^{-1}, 112 s^{-1} and 7.3 s^{-1}, respectively.

THBE had previously been shown to severely inhibit all three extradiol dioxygenases, however, no indications on the effectivity of this inactivation have been given (D'Enza, 2002). Also 3-chlorocatechol has been described as an effective inhibitor of extradiol dioxygenases (Bartels *et al.*, 1984; Vaillancourt *et al.*, 2002). Both substrates usually inactivated the enzymes too efficiently for the cleavage of these substrates to be directly monitored using spectrophotometric assays. For this reason, the inactivation of DbfB, Edo2 and Edo3 extradiol dioxygenases was quantified using DHB as reporter substrate.

The data obtained confirmed that THBE is an effective inhibitor for all three extradiol dioxygenases (Fig. 3.35).

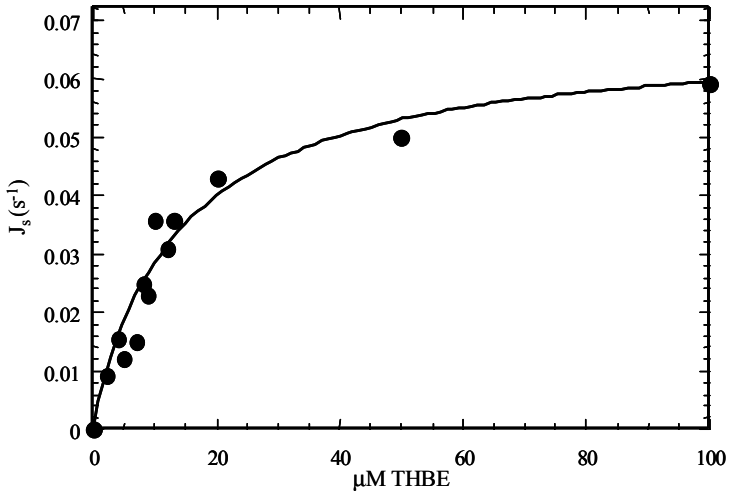

Fig. 3.35. The inactivation of extradiol dioxygenase Edo2 from *Sphingomonas wittichii* RW1 by 2,2′,3-trihydroxybiphenyl ether (THBE). Edo2 was incubated with 2,3-dihydroxybiphenyl (100 µM) and THBE in concentrations of 4 – 100 µM. The appearance of DHB ring-cleavage product was monitored at 434 nm and the rate constant of inactivation J_s at each substrate concentration of THBE was determined as described in Materials and Methods. The rate constant of inactivation J and the K_m value for THBE were calculated by fitting equation 3 to the data using KaleidaGraph. The fitted parameters are J = 0.071 ± 0.007 s^{-1} and K_m = 7.5 ± 1.5 µM.

The apparent K_m values for THBE (see Table 3.4) varied between 3 and 16.4 µM indicating all enzymes to have high affinity for this substrate. The apparent inactivation constants were also in the same order of magnitude (0.046 s^{-1} – 0.608 s^{-1}). Based on J/ K_m, the efficiency of inactivation of DbfB is similar to that of inactivation of 2,3-dihydroxybiphenyl 1,2-dioxygenase of *Burkholderia xenovorans* LB400 by 3-chlorocatechol (Vaillancourt *et al.*, 2002) whereas THBE is a less efficient inactivator for Edo2 and Edo3. Significant differences were observed regarding the inactivation by 3-chlorocatechol. Only faint amounts of 3-chlorocatechol as low as 50 nM had a dramatic effect on DHB transformation (100 µM) by Edo2.

Table 3.4. Kinetic parameters and inactivation parameters of DbfB, Edo2 and Edo3 for 2,2′,3-trihydroxybiphenylether (THBE) and 3-chlorocatechol (3CC)

Substrate	Enzyme	K_m µM	k_{cat} s^{-1}	$K_{cat}/K_,$ $\mu M^{-1} s^{-1}$	Partition ratio	J $10^{-3} s^{-1}$	J/K_m $10^3 M^{-1} s^{-1}$
THBE	DbfB	3.0 ± 0.7	20	6.7	34 ± 7	608 ± 115	203
THBE	Edo2	7.5 ± 1.5	61	8.1	856 ± 135	71 ± 7	9.5
THBE	Edo3	16.4 ± 2.6	7	0.4	154 ± 33	46 ± 3	2.8
3CC	DbfB	1200 ± 300	ND	ND	<5	32000 ± 11000	27
3CC	Edo2	0.3 ± 0.1	ND	ND	ND	180 ± 40	600
3CC	Edo3	450 ± 80	ND	ND	<30	ND	ND
3CC	BphC	4.8 ± 0.7	1.6	0.8	8 ± 2	501	104

Values were calculated by multiplying the partition ratio by J to obtain k_{cat} and by dividing the calculated k_{cat} by K_m.

In fact, inactivation experiments revealed a K_m value of 0.3 µM and based on a J/K_m value of 600 $mM^{-1} s^{-1}$, it can be assumed that efficiency of inactivation of Edo2 by 3-chlorocatechol is more pronounced then that of BphC by this substrate. However, the effects of 3-chlorocatechol on DbfB and Edo3 were much less evident. In case of Edo3, no significant inactivation was observed, even when the transformation of DHB (50 µM) was assessed in the presence of 800 µM of 3-chlorocatechol.

However, 3-chlorocatechol behaved like a mixed type inhibitor and fitting the data by non-linear regression (kindly performed by Prof. J. Bode, HZI using the programme EnzKin) revealed a competitive inhibition constant of 450 ±80 µM and an uncompetitive inhibition constant of 1150 ± 170 µM. Inhibition of DbfB was observed, however, 3-chlorocatechol obviously binds only poorly to the enzyme as revealed by the high K_m value of > 1 mM.

To further evaluate kinetic properties of DbfB, Edo 2 and Edo3, the partition ratios (the molecules of substrates that can be transformed by an enzyme molecule before complete inactivation) were determined by HPLC. Based on the amount of THBE remaining in reaction mixtures containing initially 100 µM, the partition ratio of DbfB with THBE was calculated as low as 34. Significantly higher partition ratios with THBE were observed for Edo2 (856) and Edo3 (154). In all cases, THBE was transformed into equimolar amounts of 2-pyrone-6-carboxylate. Catechol was produced as intermediate, but further transformed by the enzymes. No transformation was observed with 3-chlorocatechol, even when amounts of DbfB, Edo2 or Edo3 corresponding to 7.4 µM, 0.55 µM or 0.33 µM, respectively were incubated for up to 60 minutes with 3-chlorocatechol (100 µM).

3.3 Kinetic properties of Edo4 extradiol dioxygenase from *Sphingomonas wittichii* RW1

Activities in cell extracts of *E. coli* JM109 expressing Edo4, were significantly (by one order of magnitude) lower than the activity observed with DbfB, Edo2 or Edo3 and accounted for 190 ± 40 U/g protein.

The catalytic constants were determined directly in cell extracts comprising 20 - 35 g protein/l. 2,3-Dihydroxybiphenyl could be identified as the preferred substrate with a K_m of 1.0 ± 0.1 µM. The K_m value with THB was even lower (0.4± 0.1µM, however, V_{max} was only 28% that with DHB as substrate. Edo4 resembled Edo2 by exhibiting reasonable activities with catechol derivatives and V_{max} values with catechol were of the same order of magnitude as that with DHB (133% and 79% with catechol and 3-methylcatechol, respectively), however, relatively high K_m values were observed (217 ± 21 µM and 40 ± 4 µM with catechol and 3-methylcatechol respectively (Fig. 3.36).

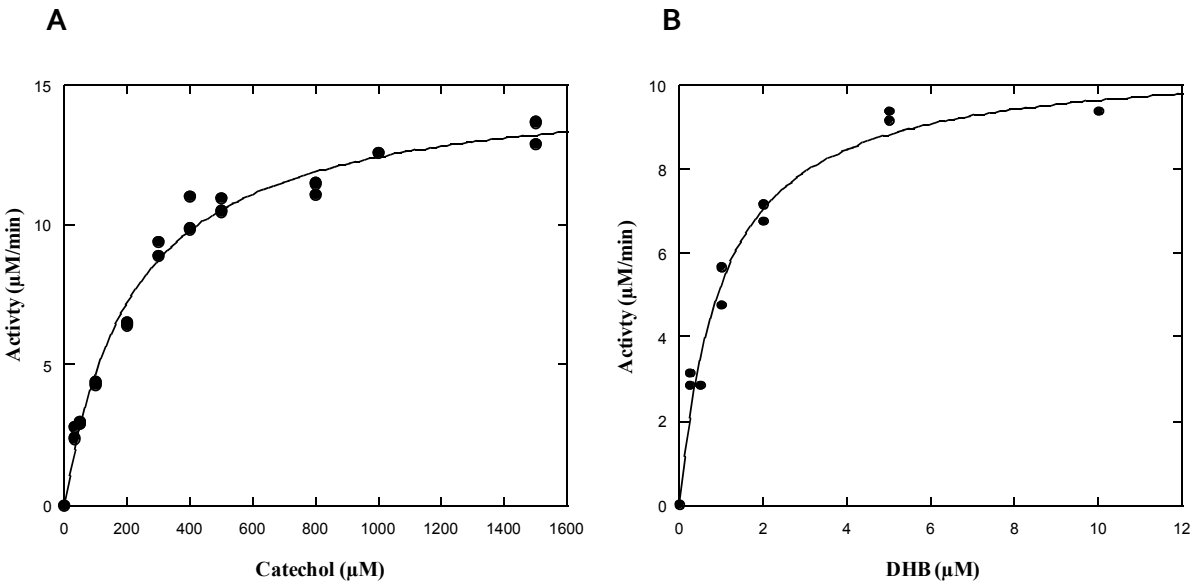

Fig. 3.36. Michaelis-Menten plot of Edo4 activity with catechol (A), and 2,3-dihydroxybiphenyl (DHB, B). The fitted parameters for catechol are K_m = 217 ± 21 µM, and V_{max} = 15.14 ± 0.48 µM/min and for 2,3-dihydroxybiphenyl K_m = 1.0 ± 0.14 µM, and V_{max} = 10.61 ± 0.44 µM/min.

The relatively low activity in cell extracts indicated, that Edo4 was not overexpressed, and in fact, no additional protein band of the expected size (33.1 kDa) was observed when cell extracts of E.coli JM109 (pEdo4) were compared by SDS page.

Therefore, a partial purification was attempted to receive indications on turnover numbers of Edo4 with its preferred substrates. The Edo4 protein exhibited a significant interaction with the column material during hydrophobic interaction chromatography. When a total activity of 2.4 U (activity against DHB) was applied to HIC, a total of 0.36 U could be recovered, probably due to partial inactivation of the enzyme during purification.

A protein band observed after SDS-Page of a molecular mass of 33 ± 2 kDa was subject to N-terminal sequencing and the determined N-terminus (SEISSLGYVGYSVTD) was identical to the one predicted for Edo4. Sypro ruby quantification indicates that the respective Edo4 protein comprised 8.9 % of the whole protein content in the most enriched protein fractions. The specific activity in this fraction could be calculated as 4550 U/g protein or 51.100 U/g Edo4. Assuming significant inactivation during purification, as described above, it can be assumed that active Edo4 has an activity of 340.700 U/g protein, which, based on a subunit molecular mass of 33.1 kDa would translate to a k_{cat} value of 188 s^{-1}. Further catalytic parameters of Edo4 are given in Table 3.5, indicating this enzyme to have extraordinary low K_m values with DHB and THB, but also to transform catechol and 3-methylcatechol with reasonable activity.

Table 3.5. Catalytic properties of four different 2,3-dihydroxybiphenyl 1,2-dioxygenases originating from *Sphingomonas wittichii* RW1.

Substrate	DbfB			Edo2			Edo3			Edo4		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)
DHB	8 ± 2	167 ± 5	20.8	140 ± 23	112 ± 11	0.80	55 ± 5	7.3 ± 0.4	0.13	1 ± 0.1	188 ± 8	188
THB	11 ± 1	50 ± 5	4.5	40 ± 15	31 ± 5	0.78	30 ± 5	6.6 ± 0.4	0.22	0.4 ± 0.1	53 ± 12	133
Catechol	83000 ± 4000	70 ± 26	0.0006	830 ± 270	34 ± 6	0.041	4000 ± 900	0.2 ± 0.04	0.00005	217 ± 21	250 ± 8	1.2
3-Methylcatechol	5300 ± 600	48 ± 2	0.011	410 ± 80	39 ± 4	0.095	10500 ± 1500	1.9 ± 0.2	0.00018	40 ± 4	149 ± 5	3.7

3-Chlorocatechol had a severe effect on Edo4 activity and rapidly inactivated the enzyme. In fact, 3-chlorocatechol inactivated too efficiently for kinetic parameters to be determined directly. For this reason, the K_m for this substrate was determined using DHB and catechol as reporter substrates. Similar values were obtained in both tests, indicating a low K_m value for this substrate (0.64 ± 0.15 μM when measured with catechol and 0.44 ± 0.11 when measured with DHB as reporter substrate). The apparent rate constant of inactivation was J= 240 ± 30 X $10^{-3}s^{-1}$ when measured with catechol as reporter substrate and J = 270 ± 45 X $10^{-3}s^{-1}$ when measured with DHB as reporter substrate and the J/K_m value of 375 mM^{-1} s^{-1} shows 3-chlorocatechol to be a potent inactivator for Edo4 (Fig. 3.37).

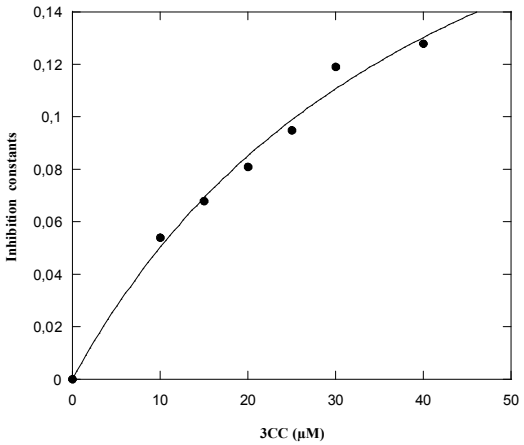

Fig. 3.37. The inactivation of extradiol dioxygenase Edo4 from *Sphingomonas wittichii* RW1 by 3-chlorocatechol (3CC). Edo4 was incubated with DHB (100 μM) and 3CC in concentrations of 10 – 40 μM. The appearance of ring-cleavage product was monitored at 434 nm and the rate constant of inactivation J_s at each concentration of 3CC was determined as described in Materials and Methods. The rate constant of inactivation J and the K_m value for 3CC were calculated by fitting equation 3 to the data using KaleidaGraph. The fitted parameters are J = 0.24 ± 0.03 s^{-1} and K_m = 0.64 ± 0.15 μM.

When kinetic parameters with THBE were to be measured with DHB as reporter substrate, only negligible enzyme inactivation was observed using THBE at concentrations up to 0.1 mM, indicating THBE to be a low affinity substrate. For this reason, kinetic parameters were measured only with catechol as reporter substrate. Analysis indicated a K_m value of 78 ± 19 μM and an apparent rate constant

of inactivation of J= 54 ± 4 X $10^{-3}s^{-1}$. Consequently a J/ K_m value of only 0.69 was obtained, which indicates THBE to be a significantly less efficient inactivator compared to 3-chlorocatechol.

HPLC based analysis of the partition ratio revealed that 3.9 nM of Edo4 were capable to transform 22 µM of THBE, pointing to a partition ratio of 5640. These data indicate Edo4 to be the most efficient extradiol dioxygenase of the four enzymes analyzed with respect to its capabilities to transform THBE.

Table 3.6. Kinetic parameters and inactivation parameters of Edo4 for 2,2´,3-trihydroxybiphenylether (THBE) and 3-chlorocatechol (3CC). Values were calculated by multiplying the partition ratio by J to obtain k_{cat} and by dividing the calculated k_{cat} by K_m.

Substrate	K_m µM	k_{cat} s^{-1}	k_{cat}/K_m $\mu M^{-1} s^{-1}$	Partition ratio	J $10^{-3} s^{-1}$	J/ K_m $10^3 M^{-1} s^{-1}$
THBE	78 ± 19	305	3.9	5640 ± 1100	54 ± 4	0.69
3CC	0.64 ± 0.15	ND	ND	ND	240 ± 30	375

4 DISCUSSION

Bacteria capable of degrading DBF are of increasing interest due to their potential to cometabolize PCDDs/PCDFs. Many bacteria that utilize DBF as a growth substrate have been isolated and characterized. Some of these such as *Sphingomonas wittichii* RW1 (Hong *et al.*, 2002; Keim *et al.*, 1999; Wilkes *et al.*, 1996) *Sphingomonas* sp. strain HL7 (Fukuda *et al.*, 2002), and *Terrabacter* sp. strain DBF63 (Habe *et al.*, 2001a), have been reported to be capable to cooxidize mono- to tetrachlorinated DD and DBF.

Several excellent reviews on bacterial DBF degradation (Bressler & Fedorak, 2000; Habe *et al.*, 2001a; Nojiri *et al.*, 2001; Wittich, 1998) appeared in the recent years, and it is generally accepted that the degradation of DBF and DD necessitates angular dioxygenation as initial reaction. In fact, a few angular dioxygenases have so far been cloned and characterized and were found to constitute novel branches in the phylogeny of Rieske non-heme iron oxygenases.

Extradiol dioxygenases play a pivotal role in numerous degradative pathways, and catalyze the second step in the DD and DBF catabolic pathway. The conversion of colorless catechol substrates to their yellow semialdehyde products (Furukawa *et al.*, 1979) has been used extensively as a convenient screening procedure for the localization and cloning of structural genes of many extradiol dioxygenases of diverse microorganisms. As for Rieske non-heme iron oxygenases, knowledge on the diversity of extradiol dioxygenases is rapidly increasing, and novel branches in the phylogeny are continuously been discovered. However, despite their importance, biochemical characterizations are still scarce and the crucial role of extradiol dioxygenases specifically for DD degradation has not been studied so far.

4.1 Isolation of DBF-utilizing *Rhodococcus* sp. strain HA01

Most of previous work to elucidate the degradation of biarylethers has been performed using *Sphingomonas* (*Sphingomonas wittichii* RW1, *Sphingomonas* sp. strain HH69) and *Terrabacter* species (*Terrabacter* sp. strain DBF63, *Terrabacter* sp. strain DPO360). However, the previous years have seen a tremendous effort in the isolation of DBF degrading microorganisms. In the present study, two microorganisms capable to mineralize DBF, i.e. *Rhodococcus* sp. strain HA01 (Fig. 4.1) and *Paenibacillus* sp. HA02 (Fig. 4.2) were isolated. Similarly, a *Paenibacillus* sp. strain YK5 was very recently isolated by the group of Kudo (Iida *et al.*, 2006). However, despite the fact that also Gram-negative organisms like *Pseudomonas* (Hong *et al.*, 2004) or *Comamonas* (Wang *et al.*, 2004) are described as being capable to degrade dibenzofuran, current literature suggests Gram-positive bacteria of the phylum *Actinobacteria.* as the most abundant culturable DBF degraders in the environment (Futamata *et al.*, 2004; Kubota *et al.*, 2005) and recently Kubota et al (Kubota *et al.*, 2005) described seven strains of DBF degrading bacteria as belonging to the new species *Nocardioides aromaticivorans*.

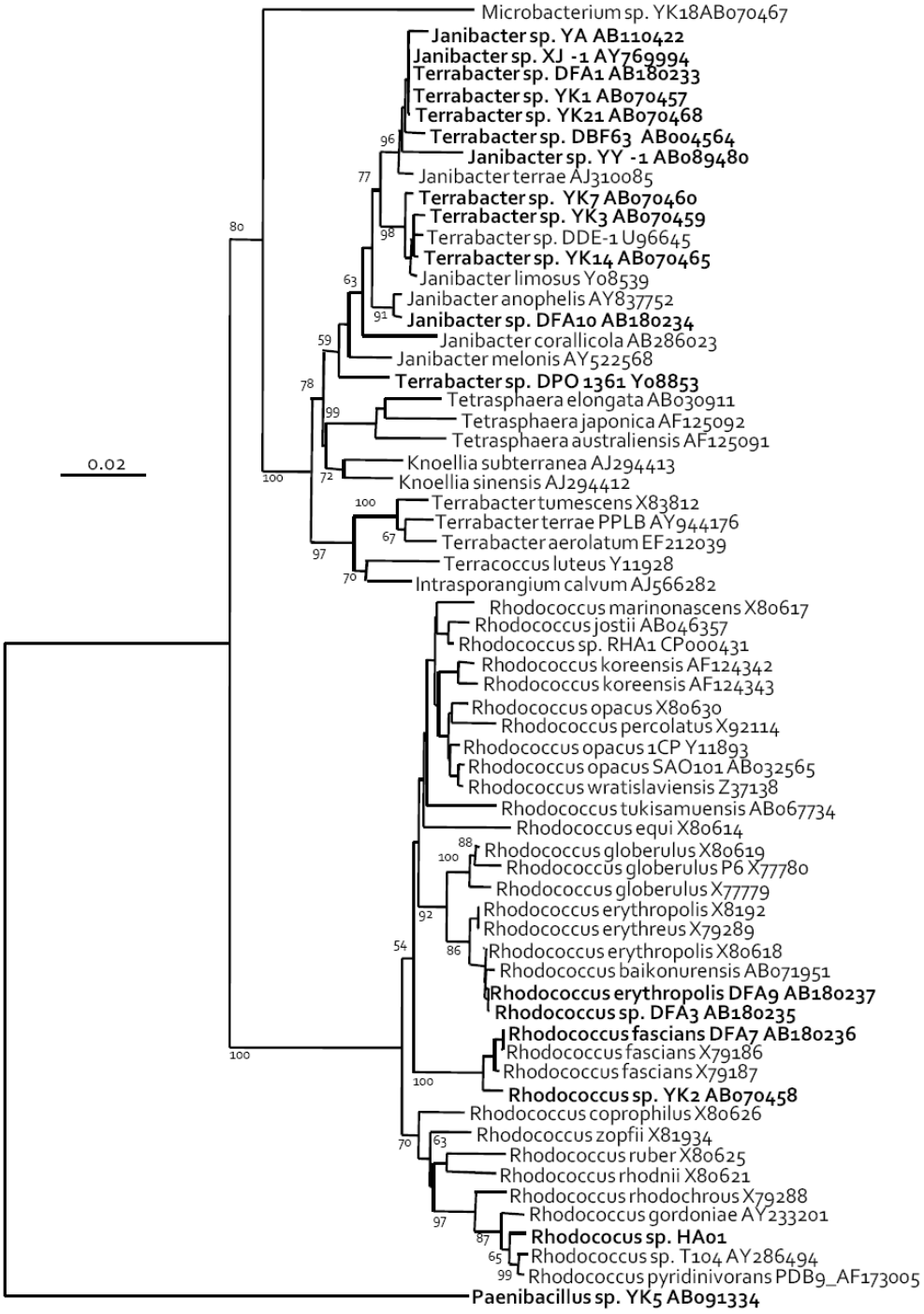

Fig. 4.1. Phylogenetic tree based on the comparison of nearly complete 16S rRNA gene sequences, showing most of the *Rhodococcus, Terrabacter* and *Janibacter* species, which can grow onDBF in bold letters and the relationship of *Rhodococcus* sp. strain HA01 with *Rhodococcus*, *Terrabacter* and *Janibacter* species. The tree was constructed by using the neighbor-joining method. Reliability of the inferred trees was tested by bootstrap using 100 resamplings. Bootstrap values beyond 50% are denoted at the branch points. The scale bar indicates 0.02 nucleotides substitutions per position. Sequence accession numbers used for phylogenetic analysis are shown after the strain name.

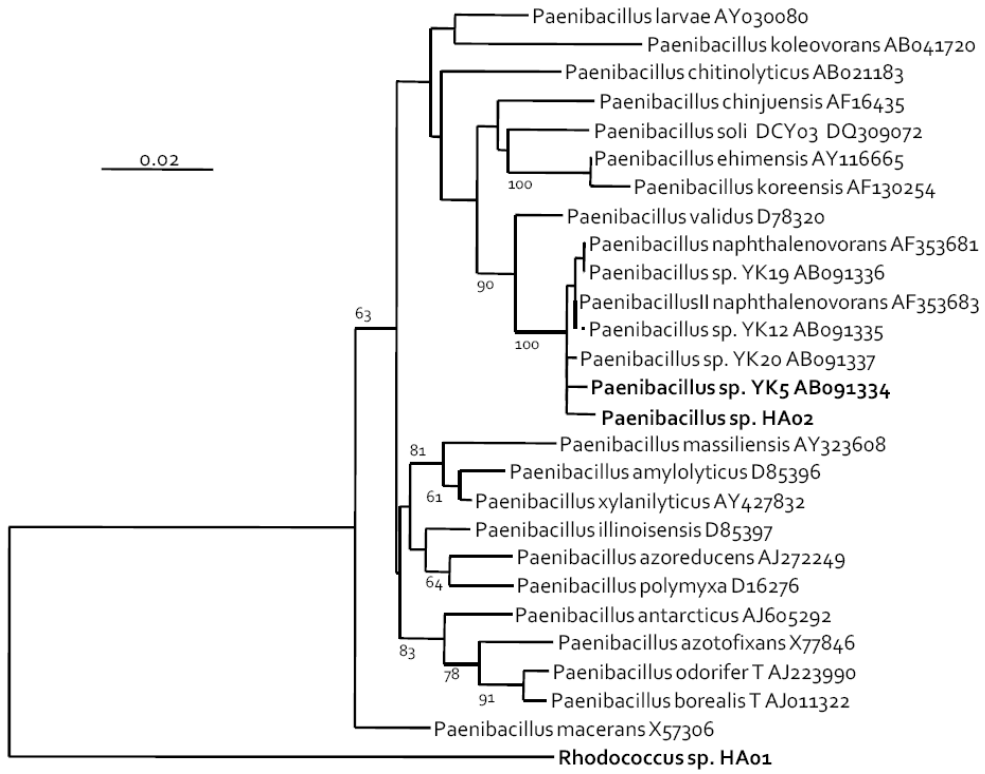

Fig. 4.2. Phylogenetic tree based on the comparison of partial 16S rRNA gene fragments, corresponding to position 530-1087 according to the *E.coli* numbering system, showing the relationship of *Paenibacillus* sp. strain HA02 with *Paenibacillus* species. The tree was constructed by using the neighbor-joining method. Reliability of the inferred trees was tested by bootstrap using 100 resamplings. Bootstrap values beyond 50% are denoted at the branch points. The scale bar indicates 0.02 nucleotides substitutions per position. Sequence accession numbers used for phylogenetic analysis are shown after the strain name.

Specifically *Janibacter* and *Terrabacter* species have often been described as capable to degrade DBF (Jin *et al.*, 2006; Yamazoe *et al.*, 2004a) Iidia et al (Iida *et al.*, 2002b) isolated 16 DBF degrading *Actinobacteria* with most of the isolates most closely related to *Terrabacter* or *Janibacter*. It should be noted, that taxonomy of bacteria closely related to *Terrabacter*/*Janibacter* is still very confusing. Based on the results of 16S rDNA analysis and DNA–DNA hybridization, Schmid et al. (Schmid *et al.*, 1997) proposed that strains DPO 360 and DPO 1361 were representatives of the same novel species within the genus *Terrabacter*. In the same year, *Terrabacter* (Collins *et al.*, 1989) was grouped with the genus *Intrasporangium* (Kalakoutskii *et al.*, 1967) in the novel family *Intrasporangiaceae* (Stackebrandt *et al.*, 1997). In the meantime, three novel genera were described within that family: *Janibacter*, (Martin *et al.*, 1997), *Terracoccus* (Prauser *et al.*, 1997) and *Tetrasphaera* (Maszenan *et al.*, 2000). *Knoellia* (Groth *et al.*, 2002) is a genus that is very close to the family *Intrasporangiaceae*. More recently, a soil isolate and two trichloroethylene-degrading strains isolated from groundwater were described, respectively, as the novel *Janibacter* species *Janibacter terrae* (Yoon *et al.*, 2000) and *Janibacter brevis* (Imamura *et al.*, 2000). DNA–DNA hybridization of two dibenzofuran-degrading strains, previously designated as *Terrabacter* sp. strain

DPO 360 and *Terrabacter* sp. strain DPO 1361 and the type strains of *J. terrae* and *J. brevis* revealed that all these four strains belong to the same species, now termed *Janibacter terrae* (Lang *et al.*, 2003). In light of these observations and supported by comparison of 16S rDNA sequence data (Fig. 4.1), it can be assumed that all DBF degrading organisms designated as *Terrabacter* or *Janibacter* species actually belong to the genus *Janibacter*.

However, also DBF degrading *Rhodococci* were recently reported and two of the 16 DBF degrading *Actinobacteria* isolated by Iida et al. (Iida *et al.*, 2002b) were most closely related to *Rhodococcus fascians*. Similarly, Noumura et al. (Noumura *et al.*, 2004) isolated, among various DBF degrading *Janibacter* species, DBF degraders closely related to *Rhodococcus fascians,* but also strains closely related to *Rhodococcus erythropolis* (Fig. 4.1).

The genus *Rhodococcus* comprise various bacteria that are capable to degrade a large number of recalcitrant organic pollutants including aromatic hydrocarbons and chlorinated aromatic compounds (de Carvalho & da Fonseca, 2005; Fuchs *et al.*, 1991; Grund *et al.*, 1992; Hara *et al.*, 2007; Heiss *et al.*, 2002; Iwabuchi *et al.*, 2002; Iwasaki *et al.*, 2006; Kim *et al.*, 2002a; Kim *et al.*, 2002b; Nandhagopal *et al.*, 2001; Navarro-Llorens *et al.*, 2005; Simoni *et al.*, 1996; Takeda *et al.*, 2004a). They achieve this through their capacity to acquire a remarkable range of diverse catabolic genes and their robust cellular physiology. It is clear that an immense gene diversity in cells that are physiologically robust could confer an advantage. As an example, the genome sequence of *Rhodococcus* sp. strain RHA1 reveals a colossal 9.7 Mb of sequence distributed between a chromosome and three large linear plasmids: pRHL1 (1100 kb), pRHL2 (450 kb) and pRHL3 (330 kb) (http://www.bcgsc.bc.ca/cgi-bin/rhodococcus/blast_rha1.pl) (McLeod *et al.*, 2006). Consistent with the genome size is a significant degree of gene redundancy and a high metabolic flexibility.

However, the relatively low levels of recent duplication and horizontal gene transfer in the chromosome of RHA1 suggest that this genome is quite stable, and that the catabolic versatility of the strain has evolved primarily through ancient acquisition or duplication processes (McLeod *et al.*, 2006). Nevertheless, the examples of functional redundancy in RHA1 central aromatic pathways suggest that selective pressure for removing such genes is low. Sequence analysis also indicated that in RHA1, the plasmids represent the most rapidly evolving parts of the genome and are important reservoirs for beneficial catabolic functions and comprise also biphenyl/PCB degradative (*bph)* genes. Notably, large linear plasmids have often been described in *Rhodococci*, and, as they usually harbor genes involved in aromatic hydrocarbon and chlorinated aromatic compounds degradation they contribute significantly to the catabolic diversity of these organisms (Larkin *et al.*, 2005).

Various *Rhodococcus* strains have been described for their capability to degrade biphenyl (*Rhodococcus erythropolis* TA421, *Rhodococcus erythropolis* TA431, *Rhodococcus rhodochrous* K37, *Rhodococcus sp*. strain HA99, *Rhodococcus* sp. Strain RHA1, *Rhodococcus globerulus* P6, *Rhodococcus opacus* SA0101 (Chung *et al.*, 1994; Iida *et al.*, 2002b; Iwasaki *et al.*, 2006; Iwasaki *et al.*, 2007; Kimura & Urushigawa,

2001; Kimura *et al.*, 2006; Maeda *et al.*, 1998; Masai *et al.*, 1995; Seto *et al.*, 1995; Warren *et al.*, 2004) and various biphenyl dioxygenases have been described from Rhodococci. However, transformation of DBF by biphenyl dioxygenases as well as naphthalene dioxygenases usually occurs by lateral oxygenation, and thus interferes with mineralization of biarylethers. In fact, Kimura et al. (Kimura *et al.*, 2006) showed expression of a naphthalene dioxygenase catalyzing lateral oxygenation of DBF and DD during growth of *Rhodococcus opacus* SAO101 on DD and suggested this enzyme to be involved in degradation. However, the strain also formed 2,2′,3-trihydroxybiphenyl ether from DD (Kimura & Urushigawa, 2001), suggesting simultaneous induction of an angular and a lateral dioxygenase in this strain, with lateral dioxygenation resulting in substrate misrouting and thus formation of dead-end metabolites. Whereas, thus genes and enzymes involved in DBF and DD degradation by SAO101 remain to be elucidated, some information was given for *Rhodococcus* sp. strain YK2 (Iida *et al.*, 2002b). However, no detailed information on the metabolic properties of a dibenzofuran degrading *Rhodococcus* were given thus far.

4.2 Degradation of DBF and DD *Rhodococcus* sp. strain HA01

DBF and DD have been used by various authors as model compounds for the investigation of the bacterial degradation of biarylethers. Even though it is believed that both DBF and DD are degraded by similar pathways, comprising initial angular dioxygenation followed by extradiol cleavage of the produced trihydroxylated intermediate and hydrolysis to produce 2-hydroxymuconate and salicylate/catechol, only a few DBF degrading bacterial strains can use DD as sole carbon source, whereas DD degraders also mineralize DBF. As examples, neither *Janibacter* YY-1 (Yamazoe *et al.*, 2004a), *Janibacter* sp. strain YA (Iwai *et al.*, 2005); *Terrabacter* sp. strain DBF63 (Monna *et al.*, 1993) nor *Sphingomonas* sp. strain HH69 (Harms *et al.*, 1990) were capable to mineralize DD. Similarly, *Rhodococcus* sp. strain HA01 grows rapidly with DBF as carbon source, however failed to mineralize DD. As discussed below, HA01 induces two angular dioxygenases to perform the initial attack on DBF and the absence of any persistence coloration resulting from the accumulation of lateral attack of the initial dioxygenase, the absence of significant amounts of intermediates as evaluated during HPLC analysis of culture supernatants and the accumulation of only 2,2′,3 trihydroxybiphenyl in the presence of 3-chlorocatechol evidences that DBF is mineralized (nearly) exclusively via angular dioxygenation. This contrasts degradation of DBF by e.g. *Nocardoides aromaticivorans* (Kubota *et al.*, 2005) *Janibacter* sp. strain YY-1 (Yamazoe *et al.*, 2004a) *Janibacter* sp. strain XJ-1 (Jin *et al.*, 2006) or *Terrabacter* sp. strain DBF63 (Monna *et al.*, 1993), which all perform significant lateral dioxygenation during growth on DBF.

Information on the reasons for the failure of DBF mineralizing strains to mineralize DD is scarce. Harms et al. (Harms *et al.*, 1990) reported *Sphingomonas* sp. strain HH69 to transform DD into a 2-phenoxy derivative of muconate, indicating extradiol ring-cleavage of intermediate 2,2′,3 trihydroxybiphenyl ether to be the crucial step. Probably, inactivation of extradiol dioxygenases during transformation of THBE as discussed below results in accumulation of THBE, which is in turn a substrate for some residual intradiol dioxygenases to produce the observed muconate intermediate. *Rhodococcus* sp. strain HA01

cannot grow on DD as a carbon source, but DBF grown cells transform DD at a rate <10% that observed with DBF as substrate without significant accumulation of intermediates. This indicates the initial angular attack to be one limiting factor in DD degradation by this strain. However, it cannot be excluded that additional factors are responsible for the failure of the strain to degrade DD.

4.3 Degradation of 3-chlorodibenzofuran and 2-chlorodibenzofuran by *Rhodococcus* sp. strain HA01

It is known that chlorinated dibenzofurans are among the most serious pollutants in the environment. Strain HA01 transforms 3CDBF nearly stoichiometrically into 4-chlorosalicylate, besides minor amounts of a yellow ring-cleavage intermediate, which are supposedly generated after lateral dioxygenation. Information on the metabolism of 3CDBF by other microorganism is scarce.

Previously, Harms et al (Harms *et al.*, 1991), revealed that a mutant of *Sphingomonas* sp. strain HH69, defective in 2,3-dihydroxybiphenyl 1,2-dioxygenase, transforms 3CDBF into equal amounts of 4'-chloro-2,2',3 trihydroxybiphenyl and 4-chloro-2,2',3 trihydroxybiphenyl indicating a non-selective attack of both the substituted and the nonsubstituted aromatic nuclei of 3-chlorodibenzofuran. Similarly, Wilkes et al. (Wilkes *et al.*, 1996) reported *Sphingomonas wittichii* RW1 to attack both the substituted and the nonsubstituted aromatic nuclei of 3-chlorodibenzofuran to form, after ring-cleavage and hydrolysis, salicylate and 4-chlorosalicylate, respectively, a situation similar to that observed in *Sphingomonas* sp. strain RW16 (Wittich *et al.*, 1999).

Evidently, the transformation of 3CDBF by *Rhododoccus* strains and HA01 analyzed here is completely different (Fig. 4.3) in that HA01 preferentially attacks the nonsubstituted aromatic nucleus of 3-CDBF. The stability of the yellow colored intermediate accounting only for a minor portion of the formed products in HA01 excludes is identity with a ring-cleavage product formed from 4-chloro-2,2',3-trihydroxybiphenyl or 4'-chloro-2,2',3 trihydroxybiphenyl as similar to the situation of the THB ring-cleavage product, the respective compounds are supposedly highly unstable and should spontaneously rearrange to form 3-(chroman-4-on-2-yl)-pyruvates(Harms *et al.*, 1991).

Also the transformation 2-chlorodibenzofuran was previously studied ((Habe *et al.*, 2001b; Parsons *et al.*, 1998; Wilkes *et al.*, 1996; Wittich *et al.*, 1999). As the case for 3CDBF, both *Sphingomonas wittichii* RW1 as well as *Sphingomonas* sp. RW16 attacked both the substituted and the nonsubstituted aromatic nuclei of 2CDBF as evidenced by the accumulation of both 5-chlorosalicylate as well as salicylate. In contrast, analysis of the sites of attack of the DFDO and CARDO angular dioxygenases of *Terrabacter* sp. strain DBF63 and *Pseudomonas resinovorans* CA10, showed angular dioxygenation to occur mainly on the nonsubstituted aromatic nucleus (Habe *et al.*, 2001a). The situation in *Rhodococcus* sp. strain HA01 again is completely different (Fig. 4.3.), as two major metabolites were formed in approximately equal amount, i.e. 5-chlorosalicylate as a result of angular attack on the non substituted aromatic nucleus and 2-chloro-3,4-dihydro-3,4-dihydroxydibenzofuran as result of lateral attack on the chlorosubstituted aromatic

nucleus. Thus, catabolic properties of HA01 differ significantly from those of previously described DBF degraders.

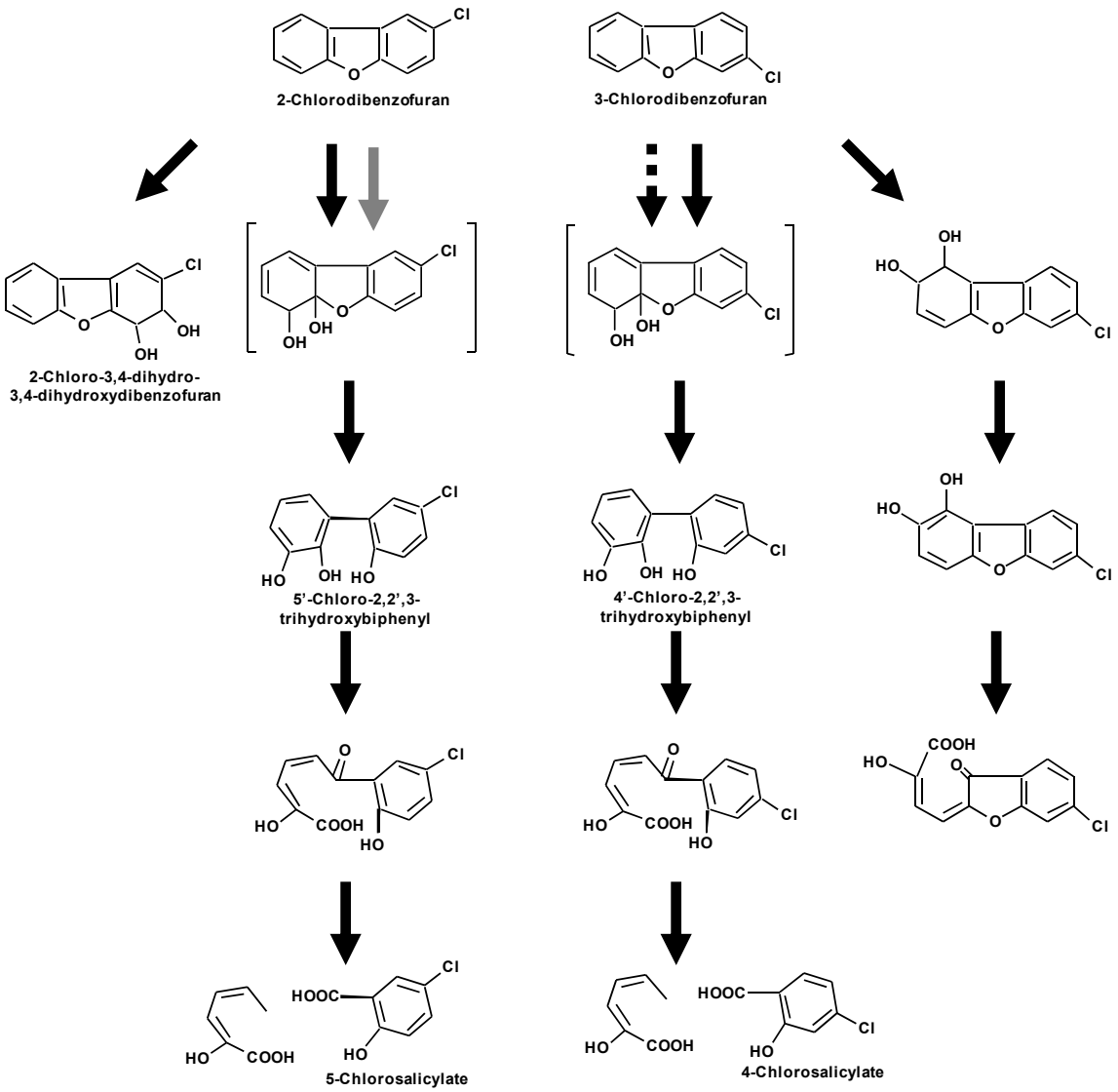

Fig. 4.3. Metabolism of 3-chlorodibenzofuran and 2-chlorodibenzofuran by *Rhodococcus* sp. strain HA01 (black arrows) and by $DbfA_{HA01}$ (grey arrow) and $DfdA_{HA01}$ (dashed black arrow) dioxygenases. Lateral dioxygenation of 3-chlorodibenzofuran by HA01 was observed to be a minor reaction, and the sites of dioxygenation are tentative.

4.4 The initial dioxygenases of *Rhodococcus* sp. strain HA01 and their function in the degradation of biarylethers

Since the initial discovery of toluene dioxygenase (TDO) by David Gibson and coworkers (Subramanian *et al.*, 1979; Yeh *et al.*, 1977), Rieske non-heme iron oxygenases have been reported to catalyze the activation of a diverse array of simple aromatic and polycyclic aromatic hydrocarbons, aromatic acids, chlorinated aromatics, or biarylethers. The involvement of angular dioxygenases in the degradation pathways for DBF and its structural analogues has been studied since two decades, however, still, only limited information is available on the diversity and distribution of such dioxygenases, their substrate range and the regioselectivity of attack. To our knowledge, angular dioxygenases have been described

from either dibenzofuran degrading organisms of from carbazole degraders but only in a few cases, a more detailed characterization is available. Among the oxygenases of DBF degraders, so far, 4 evolutionary branches can be distinguished. Dioxin 1,10'-dioxygenase (DxnA) of *Sphingomonas wittichii* RW1 (Armengaud *et al.*, 1998; Bunz & Cook, 1993) was the first angular dioxygenase to be characterized genetically and biochemically. However, substrate specificity and regioselectivity determinations for the transformation of chlorinated substrate analogues were performed using wild-type RW1, and it cannot finally be excluded, that such determinations are disturbed by the induction of additional dioxygenases. Two further types of angular dioxygenases were identified in *Terrabacter* sp. strain DBF63 (Kasuga *et al.*, 2001) (DBF 4,4'-dioxygenase, DbfA) and *Terrabacter* sp. strain YK3 (Iida *et al.*, 2002a) (DfdA). It was suggested, that enzymes similar to DfdA of *Terrabacter* sp. YK3 are distributed among dibenzofuran degrading actinobacteria. (Iida *et al.*, 2002a) A fourth evolutionary lineage was recently observed in *Paenibacillus* sp. strain YK5 (Iida *et al.*, 2006) Carbazole 1,9a-dioxygenases like the one from *Pseudomonas resinovorans* strain CA10 (Nojiri *et al.*, 1999; Sato *et al.*, 1997a; Sato *et al.*, 1997b) represent still another highly dissimilar evolutionary lineage.

Fig. 4.4. Dendrogram showing the relatedness of α-subunits of Rieske non-heme iron oxygenases. The dendrograms were calculated using tree view 1.6.6 based on sequence alignments calculated by ClustalX 1.81 using the default options. The DfdA and DbfA proteins of *Rhodococcus* sp. HA01 are shown underlined. The scale bar correspond to an estimated evolutionary distance of 0.1 amino acid substitutions per site

In the present study two angular dioxygenases were successfully localized in *Rhodococcus* sp. strain HA01, with one of them (DbfA) exhibiting high similarity to dibenzofuran dioxygenase from *Terrabacter* sp. strain DBF63 (Kasuga *et al.*, 2001), and the other one with high similarity to dibenzofuran dioxygenase DfDA from *Terrabacter* sp. strain YK3 (Iida *et al.*, 2002b) (Fig. 4.4). The existence of multiple dioxygenase genes in a single bacterium has been reported previously and seems to be common to environmental strains. For example, between four and six genes encoding for α subunits of Rieske non heme iron oxygenases were found in various species of *Sphingomonas* (Armengaud *et al.*, 1998; Kim & Zylstra, 1999; Romine *et al.*, 1999a; Romine *et al.*, 1999b; Zylstra *et al.*, 1997). It was also reported that Gram-positive bacteria contain various genes encoding Rieske non heme iron oxygenases. As an example the genome sequence of *Rhodococcus* sp. strain RHA1 revealed the presence of six ring-hydroxylating dioxygenases (McLeod *et al.*, 2006), and the dibenzofuran-utilizing organism *Rhodococcus* sp strain YK2 harbors at least four kinds of dioxygenase α-subunit genes (Iida *et al.*, 2002b). The presence of multiple Rieske non-heme iron oxygenases in *Sphingomonas* and *Rhodococcus* species may, in part at least, explain why these organisms are able to efficiently degrade a wide range of aromatic hydrocarbons. However, the presence of two distinct angular dioxygenases in a single bacterium has to our knowledge not previously been reported. Both gene clusters were expressed by HA01 in response to DBF and encode for functional enzymes, as revealed by their successful heterologous expression.

The capability of $DfdA_{YK3}$ to transform DBF by angular attack has previously been reported (Iida *et al.*, 2002a). However, the capability to transform chlorinated derivatives was not assessed previously. Analysis of the substrate specificity and regioselectivity of attack of $DfdA_{HA01}$ revealed this enzyme, like $DfdA_{YK3}$, to catalyze exclusively an angular dioxygenation of DBF. Also, like $DfdA_{YK3}$, DD was preferentially subject to angular dioxygenation, however, some lateral dioxygenation was also observed. Interestingly, $DfdA_{HA01}$ was capable to rapidly transform 3CDBF into a single metabolite, most probably 4-chloro-2,2′,3-trihydroxybiphenyl (Fig. 4.3), indicating $DfdA_{HA01}$ to significantly differ in regioselectivity from DxnA of RW1, which presumably catalyze angular dioxygenation both at the chlorosubstituted and unsubstituted nucleus (Wilkes *et al.*, 1996). Carbazol was also converted by $DfdA_{HA01}$ with the reaction product, putatively 2′-amino-2,3-dihydroxybiphenyl, being identical in spectroscopic properties with the major metabolite reported to be formed by $DfdA_{YK3}$(Iida *et al.*, 2002a). However, transformation rate of 2CDBF by $DfdA_{HA01}$, was below the detection limit and, thus, significantly lower than the transformation rate of DD, contrasting results obtained with wild-type HA01, indicating the presence of a second putatively angular dioxygenase in HA01.

In fact, expression of $DbfA_{HA01}$ revealed this enzyme to be capable of 2CDBF transformation, whereas no detectable transformation of 3CDBF was visible. However, only a single metabolite was observed from 2CDBF, which is tentatively designated as 5′-chloro-2,2′,3-trihydroxybiphenyl, contrasting the situation in wild type *Rhodococcus* sp. strain HA01, which obviously forms two distinct products, originating from angular and lateral oxygenation. It thus cannot be excluded that a third oxygenase is expressed during

growth on dibenzofuran. Taking into account the considerable amounts of lateral oxygenation observed in various DBF degrading organisms (Kubota *et al.*, 2005) (Yamazoe *et al.*, 2004a) (Jin *et al.*, 2006) (Monna *et al.*, 1993), contrasting the usually negligible lateral dioxygenation observed by cloned angular dioxygenases (Wang *et al.*, 2004) when confronted with DBF suggests, that fortuitous expression of lateral oxygenases such as naphthalene dioxygenase in *Rhodococcus opacus* SAO101 (Kimura *et al.*, 2006), is common in DBF degrading organisms. However, the extend to which substrates are misrouted by lateral dioxygenation will depend on the relative expression level and on the affinities of the expressed enzymes towards the respective substrates. Evidently, in *Rhodococcus* sp. strain HA01, the expression of two angular dioxygenase systems during growth on DBF prevents lateral oxygenation of this substrate. Moreover, it can be assumed that both angular oxygenase systems are complementary with $DfdA_{HA01}$, catalyzing 3CDBF turnover and $DbfA_{HA01}$, catalyzing 2CDBF turnover. The current observation of two specifically expressed angular oxygenases in a single host also shows that studies using wild-type organisms to reveal substrate specificities of angular dioxygenases have to be considered with care.

While pDBFR, used in the current study for heterologous expression, encodes the electron transport chain (*dfdA3A4*) of $DfdA_{HA01}$ in addition to the terminal oxygenase component (*dfdA1A2*), pDBFA12 encodes only the terminal oxygenase (*dbfA1A2*) of $DbfA_{HA01}$. Despite this lack of a primary electron transport chain, *E. coli* cells harboring pDBFA12 were capable of angular dioxygenation of DBF indicating the recruitment of host components for electron transport, Until now, there are various examples showing that electron transport can be achieved by components derived from *E. coli* host cells. As examples, *E.coli* cells containing only *dbfA1* and *dbfA2* genes encoding the large and small subunits of the dibenzofuran 4,4a-dioxygenase from *Terrabacter* sp. strain DBF63 (Kasuga *et al.*, 2001), or having received plasmids encoding naphthalene dioxygenase from *P. putida* strain NCIB9816 (Kurkela & Franck, 1990), isopropylbenzene dioxygenases from *P. putida* strain RE204 (Eaton *et al.*, 1998), or phenanthrene dioxygenase from *Burkholderia* sp. strain RP007 (Laurie & Lloyd-Jones, 1999) without a proper electron transport chain were shown to transform aromatics to their respective dihydrodiols. However, like previously reported for $DbfA_{DBF63}$, the observed activity was rather poor, and significantly lower than that of heterologously expressed $DfdA_{HA01}$. Expression of $DbfA_{HA01}$ in *Rhodococcus* sp. ATCC 12674 failed, indicating that electron transport components of this strain could not successfully be recruited. It has previously been reported that a ferredoxin encoded downstream of the *dbfA1A2* genes of *Terrabacter* sp. strain DBF63 can transfer electrons to the terminal oxygenase and that ferredoxin reductase of of phthalate dioxygenase encoded upstream of *dbfA1A2* can functionally complement an effective electron transport chain (Habe *et al.*, 2003; Takagi *et al.*, 2005) (see Fig. 1.6). As the respective gene organization in HA01 is similar to that in DBF63, it can similarly be proposed that a functional DbfA angular oxygenase also is formed in HA01 in an identical way. However, the possibility that DfdA3A4 also fulfill such a function in HA01 remains to be elucidated.

4.5 Identification of extradiol dioxygenases in *Sphingomonas wittichii* RW1

Different approaches have been used in the current study to identify extradiol dioxygenases in *Sphingomonas wittichii* RW1. Based on the fact that enzymes responsible for DBF and DD degradation in this strain were constitutively expressed (Armengaud *et al.*, 1998; D'Enza, 2002) a proteomic or transcriptomic approach was not followed. Instead, gene libraries were created to search for genes encoding extradiol dioxygenases. Phage expression libraries were found to be inadequate to search for novel genes encoding extradiol dioxygenases. Whereas the derived phagemid library proved the presence of extradiol dioxygenase encoding genes, extradiol dioxygenases could not be observed in the phage library. As extradiol dioxygenases are generally subject to inactivation in the presence of oxygen (Vaillancourt *et al.*, 1998; Vaillancourt *et al.*, 2002) it seems, that expression from phages leads to a long-term exposure to oxygen, such that activity based screening fails to detect any activity. The finally created fosmid library comprised 3000 clones, which should thus cover the whole genome of strain RW1. Even though only low expression from fosmids is expected, this approach was assumed to be the most feasible to identify novel extradiol dioxygenases, as it has been successfully used to identify extradiol dioxygenases encoding genes from an environmental *meta*-genome library (Suenaga. *et al.*, 2007). In fact, 95 out of 3000 clones from the fosmid library transformed 2,3-dihydroxybiphenyl to the yellow *meta*-cleavage product 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid). The majority of clones proved to contain the *dbfB* gene. This bias towards *dbfB* is possibly explained by the fact that genome sequencing identified *dbfB* to be located on a plasmid termed pSWIT02 (CP000701; release 92, 01-JUN-2007), contrasting previous assumptions that DBF catabolic genes were scattered on the chromosome (Armengaud *et al.*, 1998). In fact, genes previously thought to be scattered on the chromosome were localized on a 60 kb region of the 223 kb plasmid and separated by various transposases and integrases. Beside DbfB, also Edo2 and Edo5 were successfully identified by screening the genome library. However, other extradiol dioxygenase could not be identified by this screening. This is probably due to the presence of genes encoding *meta*-cleavage product hydrolases downstream of the *edo3, edo6* and *edo7* genes, which probably prevents accumulation of significant amounts of the 2,3-dihydroxybiphenyl *meta*-cleavage product. This raises some doubts on the applicability of such kind of screening to identify catabolic gene diversity in environmental samples.

In the current investigation we could show, that RW1 contains at least seven extradiol dioxygenases type I, which all exhibit activity against 2,3-dihydroxybiphenyl (see Fig. 4.5). The existence in some bacteria of multiple extradiol dioxygenases is well established (Asturias & Timmis, 1993; Kosono *et al.*, 1997; Schmid *et al.*, 1997; Taguchi *et al.*, 2004) and may indicate the capability of these strains to metabolize various aromatic compounds (Taguchi *et al.*, 2004). However, in the case of *S. wittichii* extradiol dioxygenases, the actual function of these genes can only in a few cases be deduced from the genetic environment. Edo8 has not been analyzed further in the current study, as it exhibits highest similarity (46% identity) with 2,3-dihydroxy-*p*-cumate 3,4-dioxygenase of *Pseudomonas putida* F1 (Eaton, 1996).

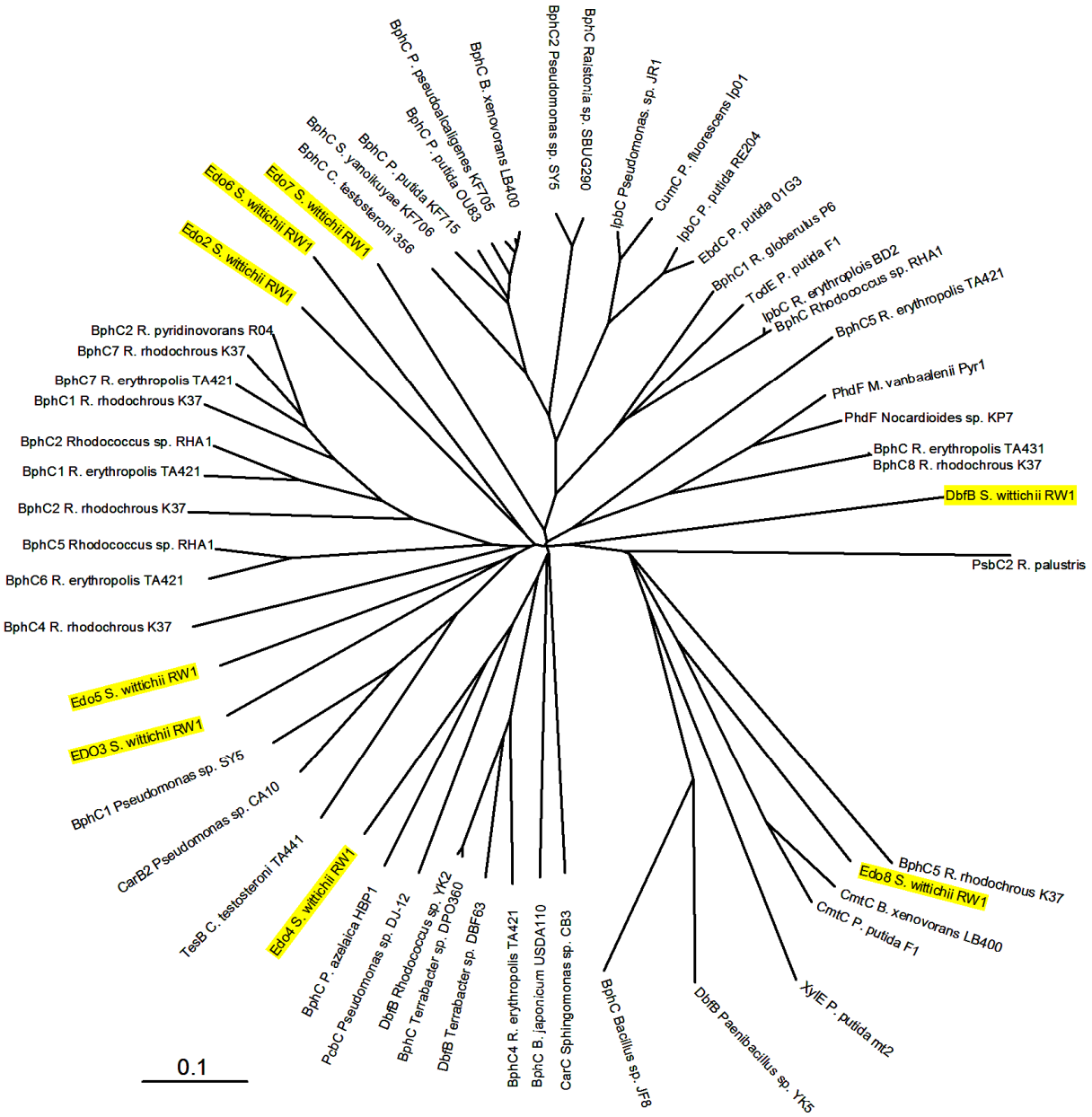

Fig. 4.5. Dendrogram showing extradiol dioxygenases type I from different bacteria including the eight extradiol dioxygenases from *S. wittichii* RW1. The dendrogram was generated using TreeView based on protein sequence alignments calculated by clustalX1.8. For accession numbers see Figure 1.8, additional accession numbers are as follows: *B. xenovorans* LB400 (CmtC, CP000270); *P. putida* F1 (CmtC, 2209341D); *Paenibacillus* sp. YK5 (DbfB, AB201843), *Bacillus* sp. JF8 (BphC), *S. wittichii* RW1 (Edo4-8, CP0000699).

Its involvement in *p*-cumate degradation is supported by the fact, that *edo8* (Swit1756) is followed by a gene, the predicted product of which exhibits 58 % of sequence identity with 2,3-dihydroxy-2,3-dihydro-p-cumate dehydrogenase of *P. putida* F1, and is preceded by genes putatively encoding for the α-and β subunits of a *p*-cumate dioxygenase (55 and 51% of sequence identity to the *P. putida* F1 gene products). As mentioned before, *edo4* (Swit3046) is followed by a gene, the product of which exhibits 63% of sequence identity with 2-hydroxybiphenyl 3-monooxygenase HpbA of *P. azelaica*. Functions of the other extradiol dioxygenases could not be deduced from the genomic context. As extradiol dioxygenases of

the type I are obviously not the crucial ones allowing RW1 to grow on DD and to effectively transform THBE, the responsible activity remains to be identified. As noted before, besides the type I extradiol dioxygenases usually involved in dibenzofuran and biphenyl degradation, type III extradiol dioxygenases belonging to the cupin superfamily and type II dioxygenases have been described.

Type II dioxygenases (Fig. 4.6) are multimers possessing one, e.g. the 2,3-dihydroxyphenylpropionate 1,2-dioxygenase (MhpB) from *E. coli* (Bugg, 1993) or two different types of subunits.

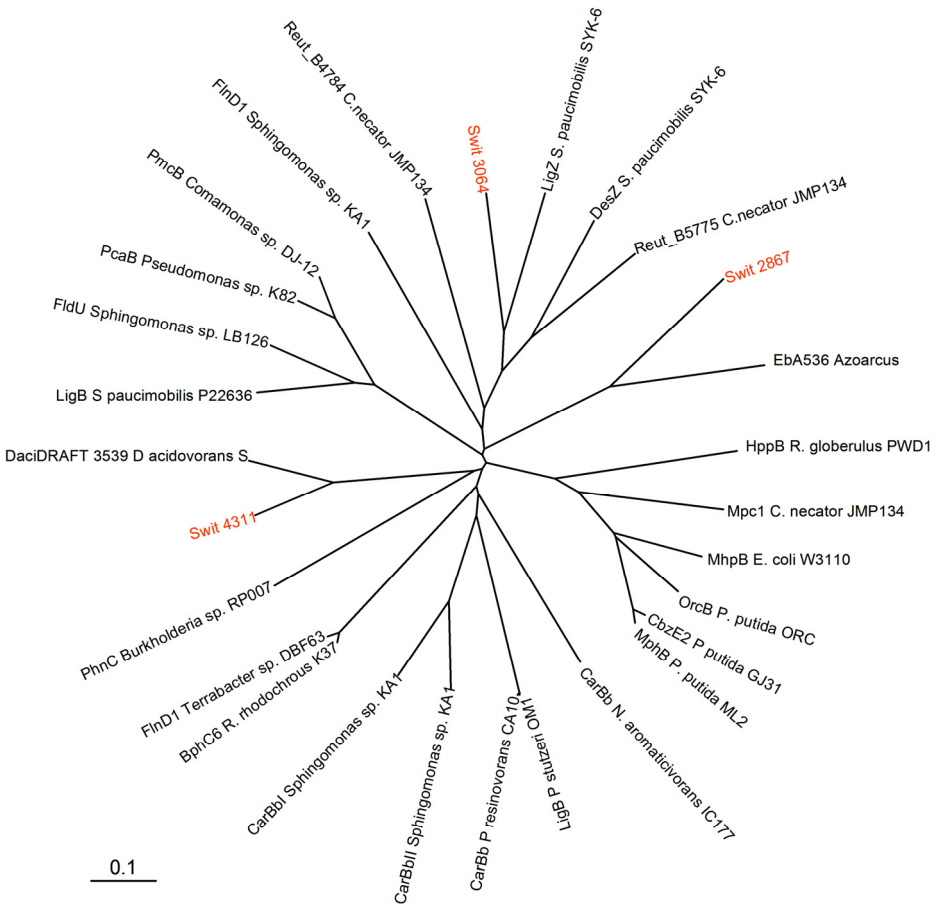

Fig. 4.6. Dendrogram showing extradiol dioxygenases type II from different bacteria including three putative extradiol dioxygenases type II from *S. wittichii* RW1. The dendrogram was generated using TreeView based on protein sequence alignments calculated by clustalX1.8. The accession numbers are as follows: *P. resinovorans* CA10 (CarBb, AB088420); *P. stutzeri* (LigB, AB001723); *Sphingomonas* sp. KA1 (CarBbII, AB220949); *Sphingomonas* sp. KA1 (CarBbI, AB095953); *N. aromaticivorans* IC177 (CarBb, AB244528); *Burkholderia* sp. RP007 (PhnC, AF061751); *R. rhodochrous* K37 (BphC6, AB117724); *Terrabacter* sp. DBF63 (FlnD1, AB095015); *S. paucimobilis* (LigB, P22636); *Sphingomonas* sp. LB126 (FldU, AJ277295); *Pseudomonas* sp. K82 (PcaB, DQ397304); *Comamonas* sp. DJ-12 (PmcB, AY866410); *Sphingomonas* sp. KA1 (FlnD1, AB270530); *D. acidovorans* SPH (DaciDRAFT_3539, NZ AAVD01000003); *.E coli* W3110 (MhpB, D86239); *P. putida* GJ31 (CbzE2, AY831461); *P. putida* ML2 (MphB, AF176355); *P. putida* ORC (OrcB, AF534914); *C. necator* JMP134 (Mpc1, P17295); *R. globerulus* PWD1 (HppB, U89712); *Azoarcus* sp. EbN1 (EbA536, NC 006513), *S. paucimobilis* SYK-6 (LigZ, AB007823); *S. paucimobilis* SYK-6 (DesZ, AB110976); *C. necator* JMP134 (Reut_B5775, Reut_B4784NC 007348); *S. wittichii* RW1 (Swit 2867, Swit 3064, Swit 4311, CP000699)

In case of the LigAB protocatechuate 4,5-dioxygenase from *Sphingomonas paucimobilis* SYK-6, which has an α2β2 composition, the large (β) and small (α) subunits appear to be unrelated, with the β subunit being similar to the protomers of the homooligomeric enzymes (Sugimoto *et al.*, 1999).

The recent years have shown that the importance of extradiol dioxygenase enzymes of class II was highly underestimated. Type II extradiol dioxygenases are involved in the degradation of gallate (Nogales *et al.*, 2005) of the lignin-related 2,2′,3-trihydroxy-3-′-methoxy-5,5′-dicarboxybiphenyl (Peng *et al.*, 1998), and in the degradation of carbazole, among others. Class II 2′-aminobiphenyl-2,3-diol 1,2-dioxygenases (consisting of α-and β subunits) have been identified to be involved in the degradation of carbazole by *P. resinovorans* CA10 (Iwata *et al.*, 2003) *Sphingomonas* sp strain KA1 (Urata *et al.*, 2006), *Nocardioides aromaticivorans* IC77 (Inoue *et al.*, 2006) or *P. stutzeri* OM1 (Ouchiyama *et al.*, 1998). Obviously, 2′-aminobiphenyl-2,3-diol 1,2-dioxygenases also catalyze the effective transformation of DHB and THB (Iwata *et al.*, 2003), however, have not yet been characterized for their capability to transform THBE.

Interestingly, protocatechuate 4,5-dioxygenase (Arciero *et al.*, 1990) was noteworthy for extreme instability as was 2′-aminobiphenyl-2,3-diol 1,2-dioxygenases from *Pseudomonas* sp. LD2 (Gibbs *et al.*, 2003). Inspection of the genome of RW1 revealed the presence of three type II dioxygenases (Swit2867, 3064, and 4311, respectively) (Fig. 4.6). Swit4311 was localized in a putative homoprotocatechuate degradative operon, whereas the putative function of Swit2867 could not be deduced by the genetic environment. Swit3064 showed highest homology (56% identity) to LigZ dioxygenase of *S. paucimobilis* SYK involved in the cleavage of lignin-related biphenyls and is thus a promising candidate to be tested for its involvement in DD degradation. In fact, the available complete genome sequence, which has just recently been assembled, will open up new avenues to characterize DD degradation by RW1.

4.6 Inactivation of extradiol dioxygenases (DbfB, Edo2, Edo3, and Edo4) from *S. wittichii* RW1 by THBE and by 3-chlorocatechol

Extradiol dioxygenases play a key role in the metabolism of dibenzofuran, dibenzo-*p*-dioxin, PCBs and various other aromatic compounds. These enzymes utilize non-heme ferrous iron to cleave the aromatic nucleus *meta* (adjacent) to the hydroxyl substituents, incorporating both atoms of dioxygen into the product. The ferrous iron of these enzymes is coordinated by two histidines and one glutamate (Han *et al.*, 1995) in what has been termed the 2-His-1-carboxylate facial triad. The ferrous center has a square pyramidal geometry with axially coordinated histidines and two solvent species as the other ligands (Han *et al.*, 1995). In the first step of the proposed mechanism, bidentate binding of the catecholic substrate displaces the two solvent ligands (Arciero & Lipscomb, 1986; Vaillancourt *et al.*, 1998) and activates the latter for O_2 binding (Arciero & Lipscomb, 1986; Shu *et al.*, 1995). Even though the subsequent steps in the catalytic mechanism are less well substantiated, biochemical studies suggest a mechanism involving iron-mediated transfer of an electron from the catechol to the O_2, yielding a semiquinone-FeII-superoxide intermediate (Spence *et al.*, 1996). This intermediate is proposed to react to give an iron-alkylperoxo intermediate which undergoes alkenyl migration, Criegee rearrangement, and O-O bond

cleavage to give an unsaturated lactone intermediate and an FeII-bound hydroxide ion, which hydrolyzes the lactone to yield the reaction product (Sanvoisin *et al.*, 1995) (Fig. 4.7).

It is known since decades that extradiol dioxygenaes may be subject to mechanism based inactivation by their aromatic substrates (Klecka & Gibson, 1981) and inactivation has initially been mainly studied using catechol 2,3-dioxygenases, specifically that of *Pseudomonas putida* mt2. Different catechols inactivate that enzyme to different extends, and the rapid inactivation by 3-chlorocatechol has been suggested to occur through covalent modification by an acyl chloride generated by ring-cleavage (Bartels *et al.*, 1984), as the enzyme could not be reactivated. However, no clear evidence for that mechanism was reported. The inactivation of catechol 2,3-dioxygenase by 4-alkylcatechols was shown to involve the accidental oxidation of the active site Fe (II) to Fe(III) during turnover (Cerdan *et al.*, 1994) and it was later on shown that various catechol *meta*-cleavage pathways have recruited a 2Fe-2S ferredoxin, which maintains the dioxygenase active site iron in the reduced state (Hugo *et al.*, 1998; Hugo *et al.*, 2000). More recent studies analyzed in detail the mechanism based inactivation of 2,3-dihydroxybiphenyl 1,2-dioxygenase from *Burkholderia xenovorans* LB400 ($BphC_{LB400}$). The analysis revealed that the inactivation was similar to the O_2-dependent inactivation of the enzyme in the absence of catecholic substrate, resulting in oxidation of the active site Fe(II) to Fe(III).

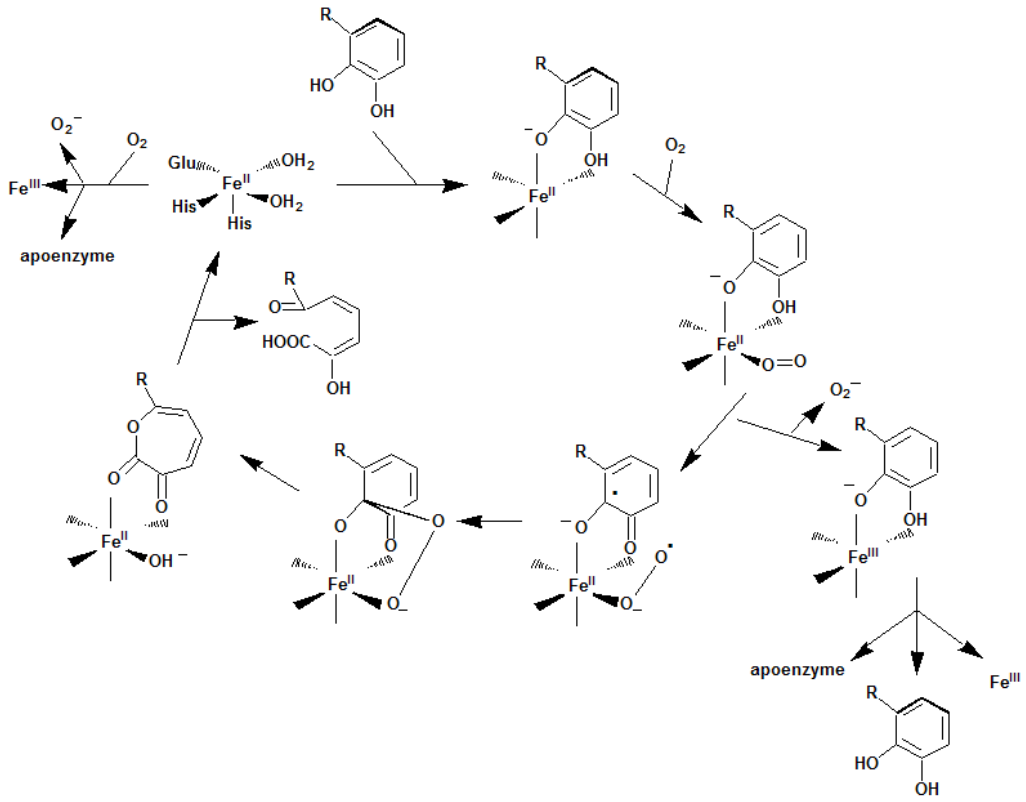

Fig. 4.7. General mechanism of inactivation of extradiol dioxygenases as described by Vaillacourt et al. (Vaillancourt *et al.*, 2002). For details, see main text.

Previous reports have shown that rapid inactivation of catechol 2,3-dioxygenases by pathway intermediates prevent the use of the substrate as growth substrate (Cerdan *et al.*, 1994; Polissi & Harayama, 1993). It is clear, that the gain of energy by metabolizing a substrate must be higher than the energy burden for resynthesis of pathway enzymes to enable growth and the rate of synthesis of catechol 2,3-dioxygenase must be higher than the rate of its inactivation. Experiments using different catechol 2,3-dioxygenase derivatives suggested that a partition ratio (the ratio of productive catalysis to suicide catalysis, or the number of substrate molecules that can be transformed by an enzyme before complete inactivation) of approximately 18.000 molecules for catechol analogues maybe a threshold allowing bacteria to grow on the respective substrates (Cerdan *et al.*, 1994). However, further experiments suggested that this threshold only holds in the presence of active ferredoxins capable to keep the enzymes in the active reduced state and for *P. putida* mt-2 the threshold value of the partition ratio required for cell growth in the absence of such a ferredoxin was calculated to be about 200,000 (Polissi & Harayama, 1993). Even though partition ratios of extradiol dioxygenases from RW1 have not been determined before, it was suggested, that the rapid observed inhibition of DbfB, Edo2 as well as of Edo3 excludes those enzymes to be of major importance for degradation of dibenzo-*p*-dioxin (D'Enza, 2002). In fact, DbfB knock-out mutants, which, like wild-type RW1 did not express Edo2 or Edo3 proteins, were still capable to degrade dibenzo-p-dioxin and dibenzofuran and the presence of a 2,3-dihydroxybiphenyl 2,3-dioxygenase activity in dibenzofuran grown cells, which could not been determined in cell free extracts, suggested that the degradation occurred via *meta*-cleavage of intermediate THB or THBE, respectively.

In the current work, we have performed a detailed analysis of the inactivation by 3-chlorocatechol, as well as THBE of 4 extradiol dioxygenases of RW1. In fact, DbfB was subject to rapid mechanism-based inactivation by THBE at extends resembling that of 2,3-dihydroxybiphenyl 1,2-dioxygenase of LB400 ($BphC_{LB400}$) by 3-chlorocatechol (Vaillancourt *et al.*, 2002). The K_m with THBE was even lower than that for the THB and DHB substrates, and K_{cat} turnover rates were only slightly lower then that for DHB or THB. Overall, DHB was shown to be the substrate preferred by the enzyme, however, there was no significant difference between THB and THBE regarding substrate preference. However, only THBE dramatically inactivates the enzyme.

The mechanism-based inactivation of extradiol dioxygenases was recently explained by dissociation of superoxide from the Enzyme-Substrate-Oxygen ternary complex (Vaillancourt *et al.*, 2002). THBE should thus, through steric or electronic factors, facilitate either the dissociation of the bound superoxide before electron transfer from the catecholate to the iron or before C-O bond formation between the bound superoxide and semiquinone. Interestingly, not only DbfB was subject to rapid inactivation, but also, however, with different efficiency, also all other extradiol dioxygenases of RW1, which were tested in this aspect, allowing the conclusion that mechanism based inactivation is probably exerted by THBE on most bacterial extradiol dioxygenases, and that degradation of dibenzo-p-dioxin necessitates specific enzymes

or enzyme complexes capable to perform effectively the task of THB cleavage. Interestingly, out of the four isoenzymes tested, Edo4 was clearly superior to DbfB, Edo2 and Edo3 for THBE transformation, as reflected by the relatively high partition ratio of 5640, and the comparably low efficiency of inactivation (J/K_m = 0.69 $mM^{-1}s^{-1}$), even though Edo4 was evidently not induced during growth on dibenzo-p-dioxin. In contrast DbfB, among the four enzymes tested the most inefficient enzyme for THBE transformation was involved in the degradation of DBF (Happe *et al.*, 1993).

The enzymes tested behaved also very differently with respect to their inactivation by 3-chlorocatechol. Such inactivation was assumed to be of environmental importance, for example in the case of 3-chlorobiphenyl transformation. 3-Chlorobiphenyl, by enzymes of the biphenyl upper pathway, is transformed into 3-chlorobenzoate, which in turn, can be transformed by wide-spread benzoate dioxygenase and benzoate dihydrodiol dehydrogenase to both 3-chloro- and 4-chlorocatechol (Pieper, 2005) Specifically 3-chlorocatechol, by mechanisms discussed above, is assumed to interfere with 3-chlorobiphenyl degradation by inhibiting and inactivating 2,3-dihydroxybiphenyl dioxygenase activity for example in *C. testosteroni* B-356 (Sondossi *et al.*, 1992). There was thus a high interest to recruit or engineer 2,3-dihydroxybiphenyl 1,2-dioxygenases with enhanced resistance against 3-chlorocatechol. So far, catechol 2,3-dioxygenases resistant against 3-chlorocatechol inhibition have been described (Kaschabek *et al.*, 1998; Mars *et al.*, 1997). *P. putida* GJ31 was found to degrade chlorobenzene rapidly via 3-chlorocatechol using a *meta*-cleavage pathway (Mars *et al.*, 1997). In contrast to other catechol 2,3-dioxygenases, which are subject to inactivation, the chlorocatechol 2,3-dioxygenase (CbzE) of strain GJ31 productively converts 3-chlorocatechol (Kaschabek *et al.*, 1998). Stoichiometric displacement of chloride occurs, leading to the production of 2-hydroxymuconate, which is further converted through the *meta*-cleavage pathway. The substrate range and turnover capacity with 3-chlorocatechol were determined for CbzE and four related catechol 2,3-dioxygenases. The results showed that CbzE was the only enzyme that could productively convert 3-chlorocatechol. Besides, CbzE was less susceptible to inactivation by methylated catechols. Studies using hybrid enzymes showed that the resistance of CbzE to suicide inactivation and its substrate specificity were mainly determined by the C-terminal region of the protein, however, single aminoacid residues important for improved resistance could not be identified (Mars *et al.*, 1999). In an effort to create 2,3-dihydroxybiphenyl 1,2-dioxygenases more resistant to 3-chlorocatechol, Ohnishi et al. (Ohnishi *et al.*, 2004) performed a screening of chimeric 2,3-dihydroxybiphenyl 1,2-dioxygenases created by family shuffling of the respective genes from two *Comamonas testosteroni* strains. Even though no detailed characterization of the proteins was performed, the determined K_i values indicated variants to be more resistant by a factor of two compared to the wild-type strains. However, in that study, the authors did not differentiate between inhibition and inactivation. In fact, only two of the four extradiol dioxygenases analyzed here, namely Edo2 and Edo4, showed a high affinity towards 3-chlorocatechol as indicated by low K_m values < 1 µM, similar to the observed K_i values of DHB12Os analyzed by Ohnishi (Ohnishi *et al.*, 2004) and relatively similar to the

reported K_m of 4.8 µM for 3-chlorocatechol of $BphC_{LB400}$ (Vaillancourt *et al.*, 2002) However, the important feature exerted by 3-chlorocatechol is not the simple inhibition, but its capability of mechanism based inactivation, the efficiency of which is reflected by the J/K_m value. Thus, even though DbfB showed only poor affinity towards 3-chlorocatechol, and, according to the definition by Ohnishi (Ohnishi *et al.*, 2004) could be regarded as a 10,000-fold improved version of $BphC_{KF702}$, it is rapidly inactivated, only slightly less efficient than $BphC_{LB400}$. However, Edo3 does not show any significant mechanism-based inactivation by 3-chlorocatechol, and the K_i of 450 µM makes it a perfect candidate for being recruited for chlorobiphenyl degradation.

It is well documented, that BphC 2,3-dihydroxybiphenyl 1,2-dioxygenases are adapted to the transformation of bicyclic compounds and are only poorly active with catechol, and exhibit also only faint activity with 3-methylcatechol, which differentiate them from catechol 1,2-dioxygenases (Eltis & Bolin, 1996), which are active mainly with catechol. Two of the dioxygenases characterized here, namely Edo2 and Edo4 obviously are less restricted in their substrate preference, as indicated by a comparison of the k_{cat}/K_m values, which differed by only 1-2 orders of magnitude between the preferred bicyclic substrates THB and DHB and the less preferred substrate catechol/3-methylcatechol, whereas in case of DbfB and Edo3, like for example for BphC1 of *Rhodococcus globerulus* P6 and $BphC_{LB400}$, 3-4 orders of magnitude differences in k_{cat}/K_m values were observed (McKay *et al.*, 2003; Vaillancourt *et al.*, 2003). Recently, some structural determinants of the substrate selectivity of bicyclic and monocyclic extradiol dioxygenases were investigated based on mutants in $BphC_{LB400}$ (Vaillancourt *et al.*, 2005). Among the mutants a V148L derivative had increased specificity for catechol. V148 usually lines the distal ring of 2,3-dihydroxybiphenyl in 2,3-dihydroxybiphenyl 1,2-dioxygenases, whereas in catechol 2,3-dioxygenases, a larger leucine is located at the respective position (Vaillancourt *et al.*, 2005). Interestingly, both Edo2 and Edo4, which both constitute novel branches in the phylogeny of extradiol dioxygenases, comprise a leucine residue at the respective position, which is located two amino acid after a FeII coordinating Histidine residue completely conserved in all extradiol dioxygenases analyzed thus far. Probably, this residue is important for the observed relatively high activity with monocyclic substrates by optimizing the proper positioning of these substrates (Vaillancourt *et al.*, 2005).

5 REFERENCES

Abd Rahman, R. N. Z., Ghazali, F. M., Salleh, A. B. & Basri, M. (2006). Biodegradation of hydrocarbon contamination by immobilized bacterial cells. *J Microbiol* **44,** 354-359.

Adriaens, P. & Focht, D. D. (1990). Continuous coculture degradation of selected polychlorinated biphenyl congeners by *Acinetobacter* spp. in an aerobic reactor system. *Environ Sci Technol* **24,** 1042-1049.

Adriaens, P. & Gribic'-Galic' (1994). Reductive dechlorination of PCDD/Fby anaerobic cultures in sediments. *Chemosphere* **29,** 2253-2259.

Adriaens, P., Q.Fu. & Grbic'-Galic (1995). Bioavailability and transformation of highly chlorinated dibenzo-*p*-dioxins and dibenzofurans in anaerobic soils and sediments. *Environ Sci Technol* **29,** 2252-2260.

Ahmed, M. & Focht, D. D. (1973). Oxidation of polychlorinated biphenyls by *achromobacter* pCB. *Bull Environ Contam Toxicol* **10,** 70-72.

Akira, H. (2003). Biodiversity of dioxin-degrading microorganisms and potential utilization in bioremediation. *Microbes Environ* **18,** 105-125.

Albers, J. M., Kreis, I. A., Liem, A. K. & van Zoonen, P. (1996). Factors that influence the level of contamination of human milk with poly-chlorinated organic compounds. *Arch Environ Contam Toxicol* **30,** 285-291.

Aoki, Y. (2001). Polychlorinated biphenyls, polychlorinated dibenzo-*p*-dioxins, and polychlorinated dibenzofurans as endocrine disrupters: what we have learned from Yusho disease. *Environ Res* **86,** 2-11.

Arai, H., Akahira, S., Ohishi, T., Maeda, M. & Kudo, T. (1998). Adaptation of *Comamonas testosteroni* TA441 to utilize phenol: organization and regulation of the genes involved in phenol degradation. *Microbiology* **144,** 2895-2903.

Arciero, D. M. & Lipscomb, J. D. (1986). Binding of 17O-labeled substrate and inhibitors to protocatechuate 4,5-dioxygenase-nitrosyl complex. Evidence for direct substrate binding to the active site Fe2+ of extradiol dioxygenases. *J Biol Chem* **261,** 2170-2178.

Arciero, D. M., Orville, A. M. & Lipscomb, J. D. (1990). Protocatechuate 4,5-dioxygenase from *Pseudomonas testosteroni*. *Methods Enzymol* **188,** 89-95.

Arcos, J. C. & Argus., M. F. (1968). Molecular geometry and carcinogenic activity of aromatic compounds. *Adv Cancer Res* **11,** 305–471.

Arfmann, H., Timmis, K. N. & Wittich, R. (1997). Mineralization of 4-chlorodibenzofuran by a consortium consisting of *Sphingomonas* sp. strain RW1 and *Burkholderia* sp. strain JWS. *Appl Environ Microbiol* **63**, 3458-3462.

Armengaud, J. & Timmis, K. N. (1997). Molecular characterization of Fdx1, a putidaredoxin-type [2Fe-2S] ferredoxin able to transfer electrons to the dioxin dioxygenase of *Sphingomonas* sp. RW1. *Eur J Biochem* **247**, 833-842.

Armengaud, J., Happe, B. & Timmis, K. N. (1998). Genetic analysis of dioxin dioxygenase of *Sphingomonas* sp. strain RW1: catabolic genes dispersed on the genome. *J Bacteriol* **180**, 3954-3966.

Armengaud, J., Timmis, K. N. & Wittich, R. M. (1999). A functional 4-hydroxysalicylate/hydroxyquinol degradative pathway gene cluster is linked to the initial dibenzo-*p*-dioxin pathway genes in *Sphingomonas* sp. strain RW1. *J Bacteriol* **181**, 3452-3461.

Asturias, J. A. & Timmis, K. N. (1993). Three different 2,3-dihydroxybiphenyl-1,2-dioxygenase genes in the gram-positive polychlorobiphenyl-degrading bacterium *Rhodococcus globerulus* P6. *J Bacteriol* **175**, 4631-4640.

Asturias, J. A., Eltis, L. D., Prucha, M. & Timmis, K. N. (1994). Analysis of three 2,3-dihydroxybiphenyl 1,2-dioxygenases found in *Rhodococcus globerulus* P6. Identification of a new family of extradiol dioxygenases. *J Biol Chem* **269**, 7807-7815.

Atlas, R. M. & Bartha, R. (1992). Hydrocarbon biodegradation and oil spill bioremediation. *Adv Microb Ecol* **12**, 287- 338.

Bajanowski, T., Furst, P., Wilmers, K., Beike, J., Kohler, H. & Brinkmann, B. (2002). Dioxin in infants--an environmental hazard? *Int J Legal Med* **116**, 27-32.

Bartels, I., Knackmuss, H. J. & Reineke, W. (1984). Suicide inactivation of catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2 by 3-halocatechols. *Appl Environ Microbiol* **47**, 500-505.

Batie, C. J., Ballou., D. P. & Correll., C. C. (1991). Phthalate dioxygenase reductase and related flavin-iron-sulfur containing electron transferases. In *Chemistry and biochemistry of flavoenzymes*, pp. 543–556. Edited by F. Muller. Boca Raton, Fla: CRC Press, Inc.

Beck, H., Dross, A. & Mathar, W. (1994). PCDD and PCDF exposure and levels in humans in Germany. *Environ Health Perspect* **102**, 173-185.

Bedard, D. L. & Van Dort, H. M. (1997). The role of microbial PCB dechlorination in natural restoration and bioremediation. *Biotechnology in the Sustainable Environment* Plenum Publishing Corp., New York. 65–71

Beil, S., Mason, J. R., Timmis, K. N. & Pieper, D. H. (1998). Identification of chlorobenzene dioxygenase sequence elements involved in dechlorination of 1,2,4,5-tetrachlorobenzene. *J Bacteriol* **180**, 5520-5528.

Bellin, J. S. & Barnes, D. G. (1985). Health hazard assessment for chlorinated dioxins and dibenzofurans other than 2, 3, 7, 8-TCDD. *Toxicol Ind Health* **1,** 235-248.

Benedik, M. J., Gibbs, P. R., Riddle, R. R. & Willson, R. C. (1998). Microbial denitrogenation of fossil fuels. *Trends Biotechnol* **16,** 390-395.

Benson, D. A., Boguski, M. S., Lipman, D. J., Ostell, J., Ouellette, B. F., Rapp, B. A. & Wheeler, D. L. (1999). GenBank. *Nucleic Acids Res* **27**, 12-17.

Bertini, I., Capozzi, F., Dikiy, A., Happe, B., Luchinat, C. & Timmis, K. N. (1995). Evidence of histidine coordination to the catalytic ferrous ion in the ring-cleaving 2,2',3-trihydroxybiphenyl dioxygenase from the dibenzofuran-degrading bacterium *Sphingomonas* sp. strain RW1. *Biochem Biophys Res Commun* **215,** 855-860.

Bianchi, D., Bosetti, A., Cidaria, D., Bernardi, A., Gagliardi, I. & D'Amico, P. (1997). Oxidation of polycyclic aromatic heterocycles by *Pseudomonas fluorescens* TTC1. *Appl Microbiol Biotechnol* **47**, 596-599

Boldt, Y. R., Sadowsky, M. J., Ellis, L. B., Que, L., Jr. & Wackett, L. P. (1995). A manganese-dependent dioxygenase from *Arthrobacter globiformis* CM-2 belongs to the major extradiol dioxygenase family. *J Bacteriol* **177,** 1225-1232.

Boldt, Y. R., Whiting, A. K., Wagner, M. L., Sadowsky, M. J., Que, L., Jr. & Wackett, L. P. (1997). Manganese(II) active site mutants of 3,4-dihydroxyphenylacetate 2,3-dioxygenase from *Arthrobacter globiformis* strain CM-2. *Biochemistry* **36,** 2147-2153.

Boyle, A. W., Phelps, C. D. & Young, L. Y. (1999). Isolation from estuarine sediments of a *Desulfovibrio* strain which can grow on lactate coupled to the reductive dehalogenation of 2,4, 6-tribromophenol. *Appl Environ Microbiol* **65,** 1133-1140.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72,** 248-254.

Brakstad, O. G. & Bonaunet, K. (2006). Biodegradation of petroleum hydrocarbons in seawater at low temperatures (0-5 degrees C) and bacterial communities associated with degradation. *Biodegradation* **17,** 71-82.

Bressler, D. C. & Fedorak, P. M. (2000). Bacterial metabolism of fluorene, dibenzofuran, dibenzothiophene, and carbazole. *Can J Microbiol* **46,** 397-409.

Brewster, D. W., Elwell, M. R. & Birnbaum, L. S. (1988). Toxicity and disposition of 2,3,4,7,8-pentachlorodibenzofuran (4PeCDF) in the rhesus monkey (*Macaca mulatta). Toxicol Appl Pharmacol* **93,** 231-246.

Brodkorb, T. S. & Legge, R. L. (1992). Enhanced biodegradation of phenanthrene in oil tar-contaminated soils supplemented with *Phanerochaete chrysosporium*. *Appl Environ Microbiol* **58,** 3117-3121.

Brown, D. P. (1987). Mortality of workers exposed to polychlorinated biphenyls--an update. *Arch Environ Health* **42**, 333-339.

Buckland, B. C., Drew, S. W., Connors, N. C. & other authors (1998). Microbial conversion of indene to indandiol: a key intermediate in the synthesis of CRIXIVAN. *Metab Eng* **1**, 63-74.

Bugg, T. D. (1993). Overproduction, purification and properties of 2,3-dihydroxyphenylpropionate 1,2-dioxygenase from *Escherichia coli*. *Biochim Biophys Acta* **1202**, 258-264.

Bunge, M., Adrian, L., Kraus, A., Opel, M., Lorenz, W. G., Andreesen, J. R., Gorisch, H. & Lechner, U. (2003). Reductive dehalogenation of chlorinated dioxins by an anaerobic bacterium. *Nature* **421**, 357-360.

Bunz, P. V. & Cook, A. M. (1993). Dibenzofuran 4,4a-dioxygenase from *Sphingomonas* sp. strain RW1: angular dioxygenation by a three-component enzyme system. *J Bacteriol* **175**, 6467-6475.

Bunz, P. V., Falchetto, R. & Cook, A. M. (1993). Purification of two isofunctional hydrolases (EC 3.7.1.8) in the degradative pathway for dibenzofuran in *Sphingomonas* sp. strain RW1. *Biodegradation* **4**, 171-178.

Button, D. K., Robertson, B. R., McIntosh, D. & Juttner, F. (1992). Interactions between marine bacteria and dissolved-phase and beached hydrocarbons after the Exxon Valdez oil spill. *Appl Environ Microbiol* **58**, 243-251.

Camara, B., Herrera, C., Gonzalez, M., Couve, E., Hofer, B. & Seeger, M. (2004). From PCBs to highly toxic metabolites by the biphenyl pathway. *Environ Microbiol* **6**, 842-850.

Camara, B. (2007). Genetic and biochemical characterisation of metabolic route observed in *Pseudomonas* sp. MT1 for the degradation of chlorosalicylates. *PhD thesis Fakultät für Lebenswissenschaften der Technischen Universität Carolo-Wilhelmina zu Braunschweig*

Cerdan, P., Wasserfallen, A., Rekik, M., Timmis, K. N. & Harayama, S. (1994). Substrate specificity of catechol 2,3-dioxygenase encoded by TOL plasmid pWWo of *Pseudomonas putida* and its relationship to cell growth. *J Bacteriol* **176**, 6074-6081.

Cerniglia, C. E., Morgan, J. C. & Gibson, D. T. (1979). Bacterial and fungal oxidation of dibenzofuran. *Biochem J* **180**, 175-185.

Chung, J. Y., Maeda, M., Song, E., Horikoshi, K. & Kudo, T. (1994). Isolation and characterization of a Gram-postive polychlorinated biphenyl-degrading bacterium, *Rhodococcus ertythropolis* strain TA421, from a termite ecosystem. *Biosci Biotechnol Biochem* **58**, 2111-2113.

Collins, M. D., Dorsch, M. & Stackebrandt, E. (1989). Transfer of *Pimelobacter tumescens* comb. nov. as *Terrabacter tumescens* comb. nov. and of *Pimelobacter jensenii* to *Nocardioides jensenii* comb. nov. *Int J Syst Bacteriol* **39**, 1-6.

Cutter, L. A., Watts, J. E., Sowers, K. R. & May, H. D. (2001). Identification of a microorganism that links its growth to the reductive dechlorination of 2,3,5,6-chlorobiphenyl. *Environ Microbiol* **3**, 699-709.

D'Enza, M. (2002). Genetic and biochemical characterization of two new extradiol dioxygenases of *Sphingomonas* sp. strain RW1 and construction of two gene cassettes for biodegradation ofdibenzofuran and dioxin. *Doctoral Thesis TU-Braunschweig*.

Daane, L. L., Harjono, I., Zylstra, G. J. & Haggblom, M. M. (2001). Isolation and characterization of polycyclic aromatic hydrocarbon-degrading bacteria associated with the rhizosphere of salt marsh plants. *Appl Environ Microbiol* **67**, 2683-2691.

Daane, L. L., Harjono, I., Barns, S. M., Launen, L. A., Palleron, N. J. & Haggblom, M. M. (2002). PAH-degradation by *Paenibacillus* spp. and description of *Paenibacillus naphthalenovorans* sp. nov., a naphthalene-degrading bacterium from the rhizosphere of salt marsh plants. *Int J Syst Evol Microbiol* **52**, 131-139.

Dahl, P., Lindstrom, G., Wiberg, K. & Rappe, C. (1995). Absorption of polychlorinated biphenyls, dibenzo-p-dioxins and dibenzofurans by breast-fed infants. *Chemosphere* **30**, 2297-2306.

Dai, S., Vaillancourt, F. H., Maaroufi, H., Drouin, N. M., Neau, D. B., Snieckus, V., Bolin, J. T. & Eltis, L. D. (2002). Identification and analysis of a bottleneck in PCB biodegradation. *Nat Struct Biol* **9**, 934-939.

de Carvalho, C. C. & da Fonseca, M. M. (2005). Degradation of hydrocarbons and alcohols at different temperatures and salinities by *Rhodococcus erythropolis* DCL14. *FEMS Microbiol Ecol* **51**, 389-399.

de Lorenzo, V., Herrero, M., Jakubzik, U. & Timmis, K. N. (1990). Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. *J Bacteriol* **172**, 6568-6572.

De Rosa, C. T., Nickle, R., Faroon, O. & Jones, D. E. (2003). The impact of toxicology on public health policy and service: an update. *Toxicol Ind Health* **19**, 115-124.

Deweerd, K. A. & Suflita, J. M. (1990). Anaerobic aryl reductive dehalogenation of halobenzoates by cell extracts of "*Desulfomonile tiedjei*". *Appl Environ Microbiol* **56**, 2999-3005.

Dunwell, J. M., Culham, A., Carter, C. E., Sosa-Aguirre, C. R. & Goodenough, P. W. (2001). Evolution of functional diversity in the cupin superfamily. *Trends Biochem Sci* **26**, 740-746.

Eaton, R. W. (1996). *p*-Cumate catabolic pathway in *Pseudomonas putida* F1: cloning and characterization of DNA carrying the *cmt* operon. *J Bacteriol* **178**, 1351-1362.

Eaton, R. W., Selifonova, O. V. & Gedney, R. M. (1998). Isopropylbenzene catabolic pathway in *Pseudomonas putida* RE204: nucleotide sequence analysis of the *ipb* operon and neighboring DNA from pRE4. *Biodegradation* **9**, 119-132.

Eltis, L. D., Hofmann, B., Hecht, H. J., Lunsdorf, H. & Timmis, K. N. (1993). Purification and crystallization of 2,3-dihydroxybiphenyl 1,2-dioxygenase. *J Biol Chem* **268**, 2727-2732.

Eltis, L. D. & Bolin, J. T. (1996). Evolutionary relationships among extradiol dioxygenases. *J Bacteriol* **178**, 5930-5937.

Engesser, K., Fietz, W., Fischer, P., Schulte, P. & Knackmuss, H.-J. (1990). Dioxygenolytic cleavage of aryl ether bonds: 1,2-dihy-dro-1,2-dihydroxy-4-carboxybenzophenone as evidence for initial 1,2-dioxygenation in 3- and 4-carboxybiphenyl ether degradation. *FEMS Microbiol Lett* **69**, 317-322.

Engesser, K. H., Strubel, V., Christoglou, K., Fischer, P. & Rast, H. G. (1989). Dioxygenolytic cleavage of aryl ether bonds: 1,10-dihydro-1,10-dihydroxyfluoren-9-one, a novel arene dihydrodiol as evidence for angular dioxygenation of dibenzofuran. *FEMS Microbiol Lett* **53**, 205-209.

Ensley, B. D. & Gibson, D. T. (1983). Naphthalene dioxygenase: purification and properties of a terminal oxygenase component. *J Bacteriol* **155**, 505-511.

Erickson, B. D. & Mondello, F. J. (1993). Enhanced biodegradation of polychlorinated biphenyls after site-directed mutagenesis of a biphenyl dioxygenase gene. *Appl Environ Microbiol* **59**, 3858-3862.

Fadullon, F. S., Karns, J. S. & Torrents, A. (1998). Degradation of atrazine in soil by *Streptomyces*. *J Environ Sci Health B* **33**, 37-49.

Ferre-Huguet, N., Nadal, M., Schuhmacher, M. & Domingo, J. L. (2006). Environmental impact and human health risks of polychlorinated dibenzo-p-dioxins and dibenzofurans in the vicinity of a new hazardous waste incinerator: a case study. *Environ Sci Technol* **40**, 61-66.

Fortin, P. D., Lo, A. T., Haro, M. A., Kaschabek, S. R., Reineke, W. & Eltis, L. D. (2005). Evolutionarily divergent extradiol dioxygenases possess higher specificities for polychlorinated biphenyl metabolites. *J Bacteriol* **187**, 415-421.

Fortnagel, P., Harms, H., Wittich, R. M., Francke, W., Krohn, S. & Meyer, H. (1989). Cleavage of dibenzofuran and dibenzodioxin ring systems by a *Pseudomonas* bacterium. *Naturwissenschaften* **76**, 222-223.

Fortnagel, P., Harms, H., Wittich, R. M., Krohn, S., Meyer, H., Sinnwell, V., Wilkes, H. & Francke, W. (1990). Metabolism of dibenzofuran by *Pseudomona*s sp. strain HH69 and the mixed culture HH27. *Appl Environ Microbiol* **56**, 1148-1156.

Fuchs, K., Schreiner, A. & Lingens, F. (1991). Degradation of 2-methylaniline and chlorinated isomers of 2-methylaniline by *Rhodococcus rhodochrous* strain CTM. *J Gen Microbiol* **137**, 2033-2039.

Fukuda, K., Nagata, S. & Taniguchi, H. (2002). Isolation and characterization of dibenzofuran-degrading bacteria. *FEMS Microbiol Lett* **208**, 179-185.

Furukawa, K., Tonomura, K. & Kamibayashi, A. (1978). Effect of chlorine substitution on the biodegradability of polychlorinated biphenyls. *Appl Environ Microbiol* **35**, 223-227.

Furukawa, K., Tomizuka, N. & Kamibayashi, A. (1979). Effect of chlorine substitution on the bacterial metabolism of various polychlorinated biphenyls. *Appl Environ Microbiol* **38**, 301-310.

Futamata, H., Uchida, T., Yoshida, N., Yonemitsu, Y. & Hiraishi, A. (2004). Distribution of dibenzofuran-degrading bacteria in soils polluted with different levels of polychlorinated dioxins. *Microbes Environ* **19**, 172-177.

Gerlt, J. A. & Babbitt, P. C. (2001). Divergent evolution of enzymatic function: mechanistically diverse superfamilies and functionally distinct suprafamilies. *Annu Rev Biochem* **70**, 209-246.

Gibbs, P. R., Riddle, R. R., Marchal, L., Benedik, M. J. & Willson, R. C. (2003). Purification and characterization of 2'aminobiphenyl-2,3-diol 1,2-dioxygenase from *Pseudomonas* sp. LD2. *Protein Expr Purif* **32**, 35-43.

Gibson, D. T. & Parales, R. E. (2000). Aromatic hydrocarbon dioxygenases in environmental biotechnology. *Curr Opin Biotechnol* **11**, 236-243.

Grosser, R. J., Warshawsky, D. & Vestal, J. R. (1991). Indegenous and enhanced mineralization of pyrene, benzo[α]-pyrene, and carbazole in soils. *Appl Environ Microbiol* **57**, 3462-3469.

Groth, I., Schumann, P., Schutze, B., Augsten, K. & Stackebrandt, E. (2002). *Knoellia sinensis* gen. nov., sp. nov. and *Knoellia subterranea* sp. nov., two novel actinobacteria isolated from a cave. *Int J Syst Evol Microbiol* **52**, 77-84.

Grund, E., Denecke, B. & Eichenlaub, R. (1992). Naphthalene degradation via salicylate and gentisate by *Rhodococcus* sp. strain B4. *Appl Environ Microbiol* **58**, 1874-1877.

Habe, H., Chung, J. S., Lee, J. H., Kasuga, K., Yoshida, T., Nojiri, H. & Omori, T. (2001a). Degradation of chlorinated dibenzofurans and dibenzo-p-dioxins by two types of bacteria having angular dioxygenases with different features. *Appl Environ Microbiol* **67**, 3610-3617.

Habe, H., Ide, K., Yotsumoto, M., Tsuji, H., Hirano, H., Widada, J., Yoshida, T., Nojiri, H. & Omori, T. (2001b). Preliminary examinations for applying a carbazole-degrader, *Pseudomonas* sp. strain CA10, to dioxin-contaminated soil remediation. *Appl Microbiol Biotechnol* **56**, 788-795.

Habe, H., Ashikawa, Y., Saiki, Y., Yoshida, T., Nojiri, H. & Omori, T. (2002a). *Sphingomonas* sp. strain KA1, carrying a carbazole dioxygenase gene homologue, degrades chlorinated dibenzo-*p*-dioxins in soil. *FEMS Microbiol Lett* **211**, 43-49.

Habe, H., Ide, K., Yotsumoto, M., Tsuji, H., Yoshida, T., Nojiri, H. & Omori, T. (2002b). Degradation characteristics of a dibenzofuran-degrader *Terrabacter* sp. strain DBF63 toward chlorinated dioxins in soil. *Chemosphere* **48**, 201-207.

Habe, H., Miyakoshi, M., Chung, J., Kasuga, K., Yoshida, T., Nojiri, H. & Omori, T. (2003). Phthalate catabolic gene cluster is linked to the angular dioxygenase gene in *Terrabacter* sp. strain DBF63. *Appl Microbiol Biotechnol* **61**, 44-54.

Habe, H., Chung, J.-S., Kato, H., Ayabe, Y., Kasuga, K., Yoshida, T., Nojiri, H., Yamane, H. & Omori, T. (2004a). Characterization of the upper pathway genes for fluorene metabolism in *Terrabacter* sp. strain DBF63. . *J Bacteriol* **186**, 5938–5944.

Habe, H., Chung, J. S., Kato, H., Ayabe, Y., Kasuga, K., Yoshida, T., Nojiri, H., Yamane, H. & Omori, T. (2004b). Characterization of the upper pathway genes for fluorene metabolism in *Terrabacter* sp. strain DBF63. *J Bacteriol* **186**, 5938-5944.

Haigler, B. E. & Gibson, D. T. (1990a). Purification and properties of ferredoxin NAP, a component of naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816. *J Bacteriol* **172**, 465-468.

Haigler, B. E. & Gibson, D. T. (1990b). Purification and properties of NADH-ferredoxin NAP reductase, a component of naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816. *J Bacteriol* **172**, 457-464.

Halden, R. U., Halden, B. G. & Dwyer, D. F. (1999). Removal of dibenzofuran, dibenzo-p-dioxin, and 2-chlorodibenzo-p-dioxin from soils inoculated with *Sphingomonas* sp. strain RW1. *Appl Environ Microbiol* **65**, 2246-2249.

Han, S., Eltis, L. D., Timmis, K. N., Muchmore, S. W. & Bolin, J. T. (1995). Crystal structure of the biphenyl-cleaving extradiol dioxygenase from a PCB-degrading pseudomonad. *Science* **270**, 976-980.

Happe, B., Eltis, L. D., Poth, H., Hedderich, R. & Timmis, K. N. (1993). Characterization of 2,2',3-trihydroxybiphenyl dioxygenase, an extradiol dioxygenase from the dibenzofuran- and dibenzo-p-dioxin-degrading bacterium *Sphingomonas* sp. strain RW1. *J Bacteriol* **175**, 7313-7320.

Hara, H., Eltis, L. D., Davies, J. E. & Mohn, W. W. (2007). Transcriptomic analysis reveals a bifurcated terephthalate degradation pathway in *Rhodococcus* sp. strain RHA1. *J Bacteriol* **189**, 1641-1647.

Harayama, S. & Rekik, M. (1989). Bacterial aromatic ring-cleavage enzymes are classified into two different gene families. *J Biol Chem* **264**, 15328-15333.

Harayama, S., Kok, M. & Neidle, E. L. (1992). Functional and evolutionary relationships among diverse oxygenases. *Annu Rev Microbiol* **46**, 565-601.

Harms, H., Wittich, R. M., Sinnwell, V., Meyer, H., Fortnagel, P. & Francke, W. (1990). Transformation of Dibenzo-p-Dioxin by *Pseudomonas* sp. Strain HH69. *Appl Environ Microbiol* **56**, 1157-1159.

Harms, H., Wilkes, H., Sinnwell, V., Wittich, R. M., Figge, K., Francke, W. & Fortnagel, P. (1991). Transformation of 3-chlorodibenzofuran by *Pseudomonas* sp. HH69. *FEMS Microbiol Lett* **65**, 25-29.

Harms, H., Wilkes, H., Wittich, R. & Fortnagel, P. (1995). Metabolism of hydroxydibenzofurans, methoxydibenzofurans, acetoxydibenzofurans, and nitrodibenzofurans by *Sphingomonas* sp. strain HH69. *Appl Environ Microbiol* **61**, 2499-2505.

Hatta, T., Mukerjee-Dhar, G., Damborsky, J., Kiyohara, H. & Kimbara, K. (2003). Characterization of a novel thermostable Mn(II)-dependent 2,3-dihydroxybiphenyl 1,2-dioxygenase from a polychlorinated biphenyl- and naphthalene-degrading *Bacillus* sp. JF8. *J Biol Chem* **278**, 21483-21492.

Hauschild, J. E., Masai, E., Sugiyama, K., Hatta, T., Kimbara, K., Fukuda, M. & Yano, K. (1996). Identification of an alternative 2,3-dihydroxybiphenyl 1,2-dioxygenase in *Rhodococcus* sp. strain RHA1 and cloning of the gene. *Appl Environ Microbiol* **62**, 2940-2846.

Hein, P., Powlowski, J., Barriault, D., Hurtubise, Y., Ahmad, D. & Sylvestre, M. (1998). Biphenyl-associated *meta*-cleavage dioxygenases from *Comamonas testosteroni* B-356. *Can J Microbiol* **44**, 42-49.

Heiss, G., Stolz, A., Kuhm, A. E., Muller, C., Klein, J., Altenbuchner, J. & Knackmuss, H. J. (1995). Characterization of a 2,3-dihydroxybiphenyl dioxygenase from the naphthalenesulfonate-degrading bacterium strain BN6. *J Bacteriol* **177**, 5865-5871.

Heiss, G., Hofmann, K. W., Trachtmann, N., Walters, D. M., Rouviere, P. & Knackmuss, H. J. (2002). npd gene functions of *Rhodococcus* (*opacus*) *erythropolis* HL PM-1 in the initial steps of 2,4,6-trinitrophenol degradation. *Microbiology* **148**, 799-806.

Hernandez, B. S., Koh, S. C., Chial, M. & Focht, D. D. (1997). Terpene-utilizing isolates and their relevance to enhanced biotransformation of polychlorinated biphenyls in soil. *Biodegradation* **8**, 153-158.

Holliger, C., Hahn, D., Harmsen, H., Ludwig, W., Schumacher, W., Tindall, B., Vazquez, F., Weiss, N. & Zehnder, A. J. (1998). *Dehalobacter restrictus* gen. nov. and sp. nov., a strictly anaerobic bacterium that reductively dechlorinates tetra- and trichloroethene in an anaerobic respiration. *Arch Microbiol* **169**, 313-321.

Hong, H. B., Chang, Y. S., Nam, I. H., Fortnagel, P. & Schmidt, S. (2002). Biotransformation of 2,7-dichloro- and 1,2,3,4-tetrachlorodibenzo-p-dioxin by *Sphingomonas wittichii* RW1. *Appl Environ Microbiol* **68**, 2584-2588.

Hong, H. B., Nam, I. H., Murugesan, K., Kim, Y. M. & Chang, Y. S. (2004). Biodegradation of dibenzo-*p*-dioxin, dibenzofuran, and chlorodibenzo-p-dioxins by *Pseudomonas veronii* PH-03. *Biodegradation* **15**, 303-313.

Hudlicky, T., Tian, X., Konigsberger, K., Maurya, R., Rouden, J. & Fan, B. (1996). Toluene dioxygenase-mediated *cis*-dihydroxylation of aromatics in enantioselective synthesis. Asymmetric total syntheses of pancratistatin and 7-deoxypancratistatin, promising antitumor agents. *J Am Chem Soc* **118**, 10752-10765

Hudlicky, T., Gonzalez, D. & Gibson, D. (1999). Enzymatic hydroxylation of aromatics in enantioselective synthesis: expanding asymmetric methodology. *Aldrichimica Acta* **32**, 35-62.

Hugo, N., Armengaud, J., Gaillard, J., Timmis, K. N. & Jouanneau, Y. (1998). A novel -2Fe-2S- ferredoxin from *Pseudomonas putida* mt2 promotes the reductive reactivation of catechol 2,3-dioxygenase. *J Biol Chem* **273**, 9622-9629.

Hugo, N., Meyer, C., Armengaud, J., Gaillard, J., Timmis, K. N. & Jouanneau, Y. (2000). Characterization of three XylT-like [2Fe-2S] ferredoxins associated with catabolism of cresols or naphthalene: evidence for their involvement in catechol dioxygenase reactivation. *J Bacteriol* **182**, 5580-5585.

Hyo Bong, H., Yoon Seok, C., Sung Deuk, C. & Yong Ha, P. (2000). Degradation of dibenzofurane by *Pseudomonas putida* PH01. *Water Res* **34**, 2404-2407.

Iida, T., Mukouzaka, Y., Nakamura, K. & Kudo, T. (2002a). Plasmid-borne genes code for an angular dioxygenase involved in dibenzofuran degradation by *Terrabacter* sp. strain YK3. *Appl Environ Microbiol* **68**, 3716-3723.

Iida, T., Mukouzaka, Y., Nakamura, K., Yamaguchi, I. & Kudo, T. (2002b). Isolation and characterization of dibenzofuran-degrading actinomycetes: analysis of multiple extradiol dioxygenase genes in dibenzofuran-degrading *Rhodococcus* species. *Biosci Biotechnol Biochem* **66**, 1462-1472.

Iida, T., Nakamura, K., Izumi, A., Mukouzaka, Y. & Kudo, T. (2006). Isolation and characterization of a gene cluster for dibenzofuran degradation in a new dibenzofuran-utilizing bacterium, *Paenibacillus* sp. strain YK5. *Arch Microbiol* **184**, 305-315.

Imamura, Y., Ikeda, M., Yoshida, S. & Kuraishi, H. (2000). *Janibacter brevis* sp. nov., a new trichloroethylene-degrading bacterium isolated from polluted environments. *Int J Syst Evol Microbiol* **50**, 1899-1903.

Inoue, K., Habe, H., Yamane, H. & Nojiri, H. (2006). Characterization of novel carbazole catabolism genes from gram-positive carbazole degrader *Nocardioides aromaticivorans* IC177. *Appl Environ Microbiol* **72**, 3321-3329.

Iwabuchi, N., Sunairi, M., Urai, M., Itoh, C., Anzai, H., Nakajima, M. & Harayama, S. (2002). Extracellular polysaccharides of *Rhodococcus rhodochrous* S-2 stimulate the degradation of aromatic components in crude oil by indigenous marine bacteria. *Appl Environ Microbiol* **68**, 2337-2343.

Iwabuchi, T. & Harayama, S. (1998). Biochemical and molecular characterization of 1-hydroxy-2-naphthoate dioxygenase from *Nocardioides* sp. KP7. *J Biol Chem* **273**, 8332-8336.

Iwai, S., Yamazoe, A., Takahashi, R., Kurisu, F. & Yagi, O. (2005). Degradation of mono-chlorinated dibenzo-p-dioxins by *Janibacter* sp. strain YA isolated from river sediment. *Curr Microbiol* **51**, 353-358.

Iwasaki, T., Miyauchi, K., Masai, E. & Fukuda, M. (2006). Multiple-subunit genes of the aromatic-ring-hydroxylating dioxygenase play an active role in biphenyl and polychlorinated biphenyl degradation in *Rhodococcus* sp. strain RHA1. *Appl Environ Microbiol* **72**, 5396-5402.

Iwasaki, T., Takeda, H., Miyauchi, K., Yamada, T., Masai, E. & Fukuda, M. (2007). Characterization of two biphenyl dioxygenases for biphenyl/PCB degradation in a PCB degrader, *Rhodococcus* sp. strain RHA1. *Biosci Biotechnol Biochem* **71**, 993-1002.

Iwata, K., Nojiri, H., Shimizu, K., Yoshida, T., Habe, H. & Omori, T. (2003). Expression, purification, and characterization of 2'-aminobiphenyl-2,3-diol 1,2-dioxygenase from carbazole-degrader *Pseudomonas resinovorans* strain CA10. *Biosci Biotechnol Biochem* **67**, 300-307.

Jaspers, M. C. M., Suske, W. A., Schmid, A., Goslings, D. A. M., Kohler, H. P. E. & van der Meer, J. R. (2000). HbpR, a new member of the XylR/DmpR subclass within the NtrC family of bacterial transcriptional activators, regulates expression of 2-hydroxybiphenyl metabolism in *Pseudomonas azelaica* HBP1. *Journal of Bacteriology* **182**, 405-417.

Jin, S., Zhu, T., Xu, X. & Xu, Y. (2006). Biodegradation of Dibenzofuran by *Janibacter terrae* Strain XJ-1. *Curr Microbiol* **53**, 30-36.

Ju, K. S. & Parales, R. E. (2006). Control of substrate specificity by active-site residues in nitrobenzene dioxygenase. *Appl Environ Microbiol* **72**, 1817-1824.

Junca, H., Plumeier, I., Hecht, H. J. & Pieper, D. H. (2004). Difference in kinetic behaviour of catechol 2,3-dioxygenase variants from a polluted environment. *Microbiology* **150**, 4181-4187.

Kahn, P. C., Gochfeld, M., Nygren, M., Hansson, M., Rappe, C., Velez, H., Ghent-Guenther, T. & Wilson, W. P. (1988). Dioxins and dibenzofurans in blood and adipose tissue of Agent Orange-exposed Vietnam veterans and matched controls. *Jama* **259**, 1661-1667.

Kalakoutskii, L. V., Kirillova, I. P. & Krassil'nikov, N. A. (1967). A new genus of the *Actinomycetales-Intrasporangium* gen. nov. *J Gen Microbiol* **48**, 79–85.

Karmaus, W., Brooks, K. R., Nebe, T., Witten, J., Obi-Osius, N. & Kruse, H. (2005). Immune function biomarkers in children exposed to lead and organochlorine compounds: a cross-sectional study. *Environ Health* **4**, 5.

Kaschabek, S. R., Kasberg, T., Muller, D., Mars, A. E., Janssen, D. B. & Reineke, W. (1998). Degradation of chloroaromatics: purification and characterization of a novel type of chlorocatechol 2,3-dioxygenase of *Pseudomonas putida* GJ31. *J Bacteriol* **180**, 296-302.

Kasuga, K., Nojiri, H., Yamane, H., Kodama, T. & Omori, T. (1997). Cloning and characterization of genes involved in the degradation of dibenzofuran by *Terrabacter* sp. strain DBF63. *J Ferment Bioeng* **84**, 387-399.

Kasuga, K., Habe, H., Chung, J. S., Yoshida, T., Nojiri, H., Yamane, H. & Omori, T. (2001). Isolation and characterization of the genes encoding a novel oxygenase component of angular dioxygenase from the gram-positive dibenzofuran-degrader *Terrabacter* sp. strain DBF63. *Biochem Biophys Res Commun* **283,** 195-204.

Kauffmann, I. M., Schmitt, J. & Schmid, R. D. (2004). DNA isolation from soil samples for cloning in different hosts. *Appl Microbiol Biotechnol* **64,** 665-670.

Kauppi, B., Lee, K., Carredano, E., Parales, R. E., Gibson, D. T., Eklund, H. & Ramaswamy, S. (1998). Structure of an aromatic-ring-hydroxylating dioxygenase-naphthalene 1,2-dioxygenase. *Structure* **6,** 571-586.

Keim, T., Francke, W., Schmidt, S. & Fortnagel, P. (1999). Catabolism of 2,7-dichloro- and 2,4,8-trichlorodibenzofuran by *Sphingomonas* sp strain RW1. *J Ind Microbiol Biotechnol* **23,** 359-363.

Kim, D., Kim, Y. S., Kim, S. K., Kim, S. W., Zylstra, G. J., Kim, Y. M. & Kim, E. (2002a). Monocyclic aromatic hydrocarbon degradation by *Rhodococcus* sp. strain DK17. *Appl Environ Microbiol* **68,** 3270-3278.

Kim, D., Park, M. J., Koh, S. C., So, J. S. & Kim, E. (2002b). Three separate pathways for the initial oxidation of limonene, biphenyl, and phenol by *Rhodococcus* sp strain T104. *J Microbiol* **40,** 86-89.

Kim, E., Kim, Y. & Kim, C. K. (1996). Genetic structures of the genes encoding 2,3-dihydroxybiphenyl 1,2-dioxygenase and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid hydrolase from biphenyl- and 4-chlorobiphenyl-degrading *Pseudomonas* sp. strain DJ-12. *Appl Environ Microbiol* **62,** 262-265.

Kim, E. & Zylstra, G. J. (1999). Functional analysis of genes involved in biphenyl, naphthalene, phenanthrene, and m-xylene degradation by *Sphingomonas yanoikuyae* B1. *J Ind Microbiol Biotechnol* **23,** 294-302.

Kimura, N., Nishi, A., Goto, M. & Furukawa, K. (1997). Functional analyses of a variety of chimeric dioxygenases constructed from two biphenyl dioxygenases that are similar structurally but different functionally. *J Bacteriol* **179,** 3936-3943.

Kimura, N. & Urushigawa, Y. (2001). Metabolism of dibenzo-p-dioxin and chlorinated dibenzo-*p*-dioxin by a gram-positive bacterium, *Rhodococcus opacus* SAO101. *J Biosci Bioeng* **92,** 138-143.

Kimura, N., Kitagawa, W., Mori, T., Nakashima, N., Tamura, T. & Kamagata, Y. (2006). Genetic and biochemical characterization of the dioxygenase involved in lateral dioxygenation of dibenzofuran from *Rhodococcus opacus* strain SAO101. *Appl Microbiol Biotechnol* **73,** 474-484.

Kitagawa, W., Suzuki, A., Hoaki, T., Masai, E. & Fukuda, M. (2001). Multiplicity of aromatic ring hydroxylation dioxygenase genes in a strong PCB degrader, *Rhodococcus* sp. strain RHA1 demonstrated by denaturing gradient gel electrophoresis. *Biosci Biotechnol Biochem* **65,** 1907-1911.

Klecka, G. M. & Gibson, D. T. (1979). Metabolism of dibenzo[1,4]dioxan by a *Pseudomonas* species. *Biochem J* **180,** 639-645.

Klecka, G. M. & Gibson, D. T. (1980). Metabolism of dibenzo-*p*-dioxin and chlorinated dibenzo-*p*-dioxins by a *Beijerinckia* species. *Appl Environ Microbiol* **39**, 288-296.

Klecka, G. M. & Gibson, D. T. (1981). Inhibition of catechol 2,3-dioxygenase from *Pseudomonas putida* by 3-chlorocatechol. *Appl Environ Microbiol* **41**, 1159-1165.

Kohler, H. P., Schmid, A. & van der Maarel, M. (1993). Metabolism of 2,2'-dihydroxybiphenyl by *Pseudomonas* sp. strain HBP1: production and consumption of 2,2',3-trihydroxybiphenyl. *J Bacteriol* **175**, 1621-1628.

Kosono, S., Maeda, M., Fuji, F., Arai, H. & Kudo, T. (1997). Three of the seven bphC genes of *Rhodococcus erythropolis* TA421, isolated from a termite ecosystem, are located on an indigenous plasmid associated with biphenyl degradation. *Appl Environ Microbiol* **63**, 3282-3285.

Krumholz, L. R., Li, J., Clarkson, W. W., Wilber, G. G. & Suflita, J. M. (1997). Transformations of TNT and related aminotoluenes in groundwater aquifer slurries under different electron-accepting conditions. *J Ind Microbiol Biotechnol* **18**, 161-169.

Kubota, M., Kawahara, K., Sekiya, K., Uchida, T., Hattori, Y., Futamata, H. & Hiraishi, A. (2005). *Nocardioides aromaticivorans* sp. nov., a dibenzofuran-degrading bacterium isolated from dioxin-polluted environments. *Syst Appl Microbiol* **28**, 165-174.

Kumagai, S., Koda, S., Miyakita, T. & Ueno, M. (2002). Polychlorinated dibenzo-*p*-dioxin and dibenzofuran concentrations in serum samples of workers at intermittently burning municipal waste incinerators in Japan. *Occup Environ Med* **59**, 362-368.

Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* **5**, 150-163.

Kurkela, S. & Franck, M. (1990). Cloning and characterization of a cold- and ABA-inducible *Arabidopsis* gene. *Plant Mol Biol* **15**, 137-144.

Lamanda, A., Zahn, A., Roder, D. & Langen, H. (2004). Improved Ruthenium II tris (bathophenantroline disulfonate) staining and destaining protocol for a better signal-to-background ratio and improved baseline resolution. *Proteomics* **4**, 599-608.

Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic acid techniques in bacterial systematics*, pp. 115-175. Edited by E. S. a. M. Goodfellow. Chichester, United Kingdom: John Wiley and Sons.

Lang, E., Kroppenstedt, R. M., Swiderski, J., Schumann, P., Ludwig, W., Schmid, A. & Weiss, N. (2003). Emended description of *Janibacter terrae*, including ten dibenzofuran-degrading strains and *Janibacter brevis* as its later heterotypic synonym. *Int J Syst Evol Microbiol* **53**, 1999-2005.

Larkin, M. J., Kulakov, L. A. & Allen, C. C. (2005). Biodegradation and *Rhodococcus* - masters of catabolic versatility. *Curr Opin Biotechnol* **16**, 282-290.

Laurie, A. D. & Lloyd-Jones, G. (1999). The *phn* genes of *Burkholderia* sp. strain RP007 constitute a divergent gene cluster for polycyclic aromatic hydrocarbon catabolism. *J Bacteriol* **181,** 531-540.

Liu, S. & Suflita, J. M. (1993). Ecology and evolution of microbial populations for bioremediation. *Trends Biotechnol* **11,** 344-352.

Maeda, K., Nojiri, H., Shintani, M., Yoshida, T., Habe, H. & Omori, T. (2003). Complete nucleotide sequence of carbazole/dioxin-degrading plasmid pCAR1 in *Pseudomonas resinovorans* strain CA10 indicates its mosaicity and the presence of large catabolic transposon Tn4676. *J Mol Biol* **326,** 21-33.

Maeda, M., Chung, S. Y., Song, E. & Kudo, T. (1995). Multiple genes encoding 2,3-dihydroxybiphenyl 1,2-dioxygenase in the gram-positive polychlorinated biphenyl-degrading bacterium *Rhodococcus erythropolis* TA421, isolated from a termite ecosystem. *Appl Environ Microbiol* **61,** 549-555.

Maeda, M., Roberts, M. S., Ohta, Y., Fuji, F., Travisano, M. & Kudo, T. (1998). Isolation and characterization of a new aromatic compound-degrading alkalitrophic bacteria. *J Gen Appl Microbiol* **44,** 101-106.

Mars, A. E., Kasberg, T., Kaschabek, S. R., van Agteren, M. H., Janssen, D. B. & Reineke, W. (1997). Microbial degradation of chloroaromatics: use of the *meta*-cleavage pathway for mineralization of chlorobenzene. *J Bacteriol* **179,** 4530-4537.

Mars, A. E., Kingma, J., Kaschabek, S. R., Reineke, W. & Janssen, D. B. (1999). Conversion of 3-chlorocatechol by various catechol 2,3-dioxygenases and sequence analysis of the chlorocatechol dioxygenase region of *Pseudomonas putida* GJ31. *J Bacteriol* **181,** 1309-1138.

Martin, K., Schumann, P., Rainey, F. A., Schuetze, B. & Groth, I. (1997). *Janibacter limosus* gen. nov., sp. nov., a new actinomycete with meso-diaminopimelic acid in the cell wall. *Int J Syst Bacteriol* **47,** 529-534.

Masai, E., Yamada, A., Healy, J. M., Hatta, T., Kimbara, K., Fukuda, M. & Yano, K. (1995). Characterization of biphenyl catabolic genes of gram-positive polychlorinated biphenyl degrader *Rhodococcus* sp. strain RHA1. *Appl Environ Microbiol* **61,** 2079-2085.

Maszenan, A. M., Seviour, R. J., Patel, B. K., Schumann, P., Burghardt, J., Tokiwa, Y. & Stratton, H. M. (2000). Three isolates of novel polyphosphate-accumulating gram-positive cocci, obtained from activated sludge, belong to a new genus, *Tetrasphaera* gen. nov., and description of two new species, *Tetrasphaera japonica* sp. nov. and *Tetrasphaera australiensis* sp. nov. *Int J Syst Evol Microbiol* **50** 593-603.

Mayes, B. A., McConnell, E. E., Neal, B. H. & other authors (1998). Comparative carcinogenicity in Sprague-Dawley rats of the polychlorinated biphenyl mixtures Aroclors 1016, 1242, 1254, and 1260. *Toxicol Sci* **41,** 62-76.

McConnell, E. E., Moore, J. A. & Dalgard, D. W. (1978). Toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in rhesus monkeys (*Macaca mulatta*) following a single oral dose. *Toxicol Appl Pharmacol* **43,** 175-187.

McKay, D. B., Seeger, M., Zielinski, M., Hofer, B. & Timmis, K. N. (1997). Heterologous expression of biphenyl dioxygenase-encoding genes from a Gram-positive broad-spectrum polychlorinated biphenyl degrader and characterization of chlorobiphenyl oxidation by the gene products. *J Bacteriol* **179,** 1924–1930.

McKay, D. B., Prucha, M., Reineke, W., Timmis, K. N. & Pieper, D. H. (2003). Substrate specificity and expression of three 2,3-dihydroxybiphenyl 1,2-dioxygenases from *Rhodococcus globerulus* strain P6. *J Bacteriol* **185,** 2944-2951.

McLeod, M. P., Warren, R. L., Hsiao, W. W. & other authors (2006). The complete genome of *Rhodococcus* sp. RHA1 provides insights into a catabolic powerhouse. *Proc Natl Acad Sci U S A* **103,** 15582-15587.

Megharaj, M., Jovcic, A., Boul, H. L. & Thiele, J. H. (1997). Recalcitrance of 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (DDE) to cometabolic degradation by pure cultures of aerobic and anaerobic bacteria. *Arch Environ Contam Toxicol* **33,** 141-146.

Meintanis, C., Chalkou, K. I., Kormas, K. A. & Karagouni, A. D. (2006). Biodegradation of crude oil by thermophilic bacteria isolated from a volcano island. *Biodegradation* **17,** 105-111.

Miyazawa, D., Mukerjee-Dhar, G., Shimura, M., Hatta, T. & Kimbara, K. (2004). Genes for Mn(II)-dependent NahC and Fe(II)-dependent NahH located in close proximity in the thermophilic naphthalene and PCB degrader, *Bacillus* sp. JF8: cloning and characterization. *Microbiology* **150,** 993-1004.

Mohn, W. & Tiedje, J. (1991). Evidence for chemiosmotic coupling of reductive dechlorination and ATP synthesis in *Desulfomonile*. *Arch Microbiol* **157,** 1-6.

Mohn, W. W. & Tiedje, J. M. (1990). Strain DCB-1 conserves energy for growth from reductive dechlorination coupled to formate oxidation. *Arch Microbiol* **153,** 267-271.

Mondello, F. J. (1989). Cloning and expression in *Escherichia coli* of *Pseudomonas* strain LB400 genes encoding polychlorinated biphenyl degradation. *J Bacteriol* **171,** 1725-1732.

Mondello, F. J., Turcich, M. P., Lobos, J. H. & Erickson, B. D. (1997). Identification and modification of biphenyl dioxygenase sequences that determine the specificity of polychlorinated biphenyl degradation. *Appl Environ Microbiol* **63,** 3096-3103.

Monna, L., Omori, T. & Kodama, T. (1993). Microbial degradation of dibenzofuran, fluorene, and dibenzo-*p*-dioxin by *Staphylococcus auriculans* DBF63. *Appl Environ Microbiol* **59,** 285-289.

Moran, A. C., Muller, A., Manzano, M. & Gonzalez, B. (2006). Simazine treatment history determines a significant herbicide degradation potential in soils that is not improved by bioaugmentation with *Pseudomonas* sp. ADP. *J Appl Microbiol* **101,** 26-35.

Mousa, M. A., Ganey, P. E., Quensen III, J. F., Madhukar, B. V., Chou, K., Giesy, J. P., Fischer, L. J. & Boyd, S. A. (1998). Altered biologic activities of commercial polychlorinated biphenyl mixtures after microbial reductive dechlorination *Environ Health Perspect* **106** 1409–1418.

Mueller, J. G., P. J. Chapman, and P. H. Pritchard. (1989). Creosote-contaminated sites: their potential for bioremediation. *Environ Sci Technol* **23**, 1197–1201.

Mushrush, G. W., E. J. Beal, D. R. Hardy, and J. M. Hughes. (1999). Nitrogen compound distribution in middle distillate fuels derived from petroleum, oil shale, and tar sand sources. *Fuel Process Technol* **61**, 197–210.

Nam, J. W., Nojiri, H., Yoshida, T., Habe, H., Yamane, H. & Omori, T. (2001). New classification system for oxygenase components involved in ring-hydroxylating oxygenations. *Biosci Biotechnol Biochem* **65**, 254-263.

Nam, J. W., Fujimoto, Z., Mizuno, H., Yamane, H., Yoshida, T., Habe, H., Nojiri, H. & Omori, T. (2002). Crystallization and preliminary crystallographic analysis of the terminal oxygenase component of carbazole 1,9a-dioxygenase of *Pseudomonas resinovorans* strain CA10. *Acta Crystallogr D Biol Crystallogr* **58**, 1350-1352.

Nandhagopal, N., Yamada, A., Hatta, T., Masai, E., Fukuda, M., Mitsui, Y. & Senda, T. (2001). Crystal structure of 2-hydroxyl-6-oxo-6-phenylhexa-2,4-dienoic acid (HPDA) hydrolase (BphD enzyme) from the *Rhodococcus* sp. strain RHA1 of the PCB degradation pathway. *J Mol Biol* **309**, 1139-1151.

Navarro-Llorens, J. M., Patrauchan, M. A., Stewart, G. R., Davies, J. E., Eltis, L. D. & Mohn, W. W. (2005). Phenylacetate catabolism in *Rhodococcus* sp. strain RHA1: a central pathway for degradation of aromatic compounds. *J Bacteriol* **187**, 4497-4504.

Noda, Y., Nishikawa, S., Shiozuka, K. & other authors (1990). Molecular cloning of the protocatechuate 4,5-dioxygenase genes of *Pseudomonas paucimobilis*. *J Bacteriol* **172**, 2704-2709.

Nogales, B., Moore, E. R., Abraham, W. R. & Timmis, K. N. (1999). Identification of the metabolically active members of a bacterial community in a polychlorinated biphenyl-polluted moorland soil. *Environ Microbiol* **1**, 199-212.

Nogales, J., Canales, A., Jimenez-Barbero, J., Garcia, J. L. & Diaz, E. (2005). Molecular characterization of the gallate dioxygenase from *Pseudomonas putida* KT2440. The prototype of a new subgroup of extradiol dioxygenases. *J Biol Chem* **280**, 35382-35390.

Nojiri, H., Nam, J. W., Kosaka, M., Morii, K. I., Takemura, T., Furihata, K., Yamane, H. & Omori, T. (1999). Diverse oxygenations catalyzed by carbazole 1,9a-dioxygenase from *Pseudomonas* sp. Strain CA10. *J Bacteriol* **181**, 3105-3113.

Nojiri, H., Habe, H. & Omori, T. (2001). Bacterial degradation of aromatic compounds via angular dioxygenation. *J Gen Appl Microbiol* **47**, 279-305.

Nojiri, H. & Omori, T. (2002). Molecular bases of aerobic bacterial degradation of dioxins: involvement of angular dioxygenation. *Biosci Biotechnol Biochem* **66**, 2001-2016.

Noumura, T., Habe, H., Widada, J., Chung, J. S., Yoshida, T., Nojiri, H. & Omori, T. (2004). Genetic characterization of the dibenzofuran-degrading *Actinobacteria* carrying the dbfA1A2 gene homologues isolated from activated sludge. *FEMS Microbiol Lett* **239**, 147-155.

Ohnishi, K., Okuta, A., Ju, J., Hamada, T., Misono, H. & Harayama, S. (2004). Molecular breeding of 2,3-dihydroxybiphenyl 1,2-dioxygenase for enhanced resistance to 3-chlorocatechol. *J Biochem (Tokyo)* **135**, 305-317.

Otles, S. & Yildiz, H. (2003). Dioxin in food and human health. *Electron J Environ Agric Food Chem* **2**, 593-608.

Ouchiyama, N., Zhang, Y., Omori, T. & Kodama, T. (1993). Biodegradation of carbazole by *Pseudomonas* spp. CA06 andCA10. *Biosci Biotechnol Biochem* **57**, 455–460.

Ouchiyama, N., Miyachi, S. & Omori, T. (1998). Cloning and nucleotide sequence of carbazole catabolic genes from *Pseudomonas stutzeri* strain OM1, isolated from activated sludge. *J Gen Appl Microbiol* **44**, 57-63.

Parales, J. V., Parales, R. E., Resnick, S. M. & Gibson, D. T. (1998). Enzyme specificity of 2-nitrotoluene 2,3-dioxygenase from *Pseudomonas* sp. strain JS42 is determined by the C-terminal region of the alpha subunit of the oxygenase component. *J Bacteriol* **180**, 1194-1199.

Parales, R. E. (2003). The role of active-site residues in naphthalene dioxygenase. *J Ind Microbiol Biotechnol* **30**, 271-278.

Parsons, J. R., de Bruijne, J. A. & Weiland, A. R. (1998). Biodegradation pathway of 2-chlorodibenzo-p-dioxin and 2-chlorodibenzofuran in the biphenyl-utilising strain JB1. *Chemosphere* **37**, 1915-1922.

Peng, X., Egashira, T., Hanashiro, K., Masai, E., Nishikawa, S., Katayama, Y., Kimbara, K. & Fukuda, M. (1998). Cloning of a *Sphingomonas paucimobilis* SYK-6 gene encoding a novel oxygenase that cleaves lignin-related biphenyl and characterization of the enzyme. *Appl Environ Microbiol* **64**, 2520-2527.

Pfeifer, F., TruÈ per, H., Klein, J. & S., S. (1993). Degradation of diphenyl ether by *Pseudomonas cepacia* ET4: enzymatic release of phenol from 2,3-dihydroxydiphenylether. *Arch Microbiol* **159**, 323-329.

Pieper, D. H., Stadler-Fritzsche, K., Engesser, K. H. & Knackmuss, H. J. (1993). Metabolism of 2-chloro-4-methylphenoxyacetate by *Alcaligenes eutrophus* JMP 134. *Arch Microbiol* **160**, 169-178.

Pieper, D. H., Pollmann, K., Nikodem, P., Gonzalez, B. & Wray, V. (2002). Monitoring key reactions in degradation of chloroaromatics by in situ (1)H nuclear magnetic resonance: solution structures of metabolites formed from cis-dienelactone. *J Bacteriol* **184**, 1466-1470.

Pieper, D. H. (2005). Aerobic degradation of polychlorinated biphenyls. *Appl Microbiol Biotechnol* **67**, 170-191.

Polissi, A. & Harayama, S. (1993). In vivo reactivation of catechol 2,3-dioxygenase mediated by a chloroplast-type ferredoxin: a bacterial strategy to expand the substrate specificity of aromatic degradative pathways. *Embo J* **12**, 3339-3347.

Posada, A. C. (2006). Biphenyl degradation genes in *Rhodococcus* sp. I24. *MURJ* **14**, 18-21.

Potrawfke, T., Armengaud, J. & Wittich, R. M. (2001). Chlorocatechols substituted at positions 4 and 5 are substrates of the broad-spectrum chlorocatechol 1,2-dioxygenase of *Pseudomonas chlororaphis* RW71. *J Bacteriol* **183**, 997-1011.

Prauser, H., Schumann, P., Rainey, F. A., Kroppenstedt, R. M. & Stackebrandt, E. (1997). *Terracoccus luteus* gen. nov., sp. nov., an LL-diaminopimelic acid-containing coccoid actinomycete from soil. *Int J Syst Bacteriol* **47**, 1218-1224.

Que, L., Jr. & Reynolds, M. F. (2000). Manganese(II)-dependent extradiol-cleaving catechol dioxygenases. *Met Ions Biol Syst* **37**, 505-525.

Quensen, J. F., Tiedje, J. M., Boyd, S. A., Enke, C., Lopshire, R., Giesy, J. P., Mora, M. A., Crawford, R. & Tillitt, D. (1992). Evaluation of the suitability of reductive dechlorination for the bioremediation of PCB-contaminated soils and sediments In *International Symposium on Soil Decontamination using Biological Processes Karlsruhe* pp. 91–100. Frankfurt am Main: Dechema.

Quensen, J. F., Mousa, M. A., Boyd, S. A., Sanderson, J. T., Froese, K. L. & Giesy, J. P. (1998). Reduction of aryl hydrocarbon receptor-mediated activity of polychlorinated biphenyl mixtures due to anaerobic microbial dechlorination. *Environ Toxicol Chem* **17**, 806-813.

Rabilloud, T., Strub, J. M., Luche, S., van Dorsselaer, A. & Lunardi, J. (2001). A comparison between Sypro Ruby and ruthenium II tris (bathophenanthroline disulfonate) as fluorescent stains for protein detection in gels. *Proteomics* **1**, 699-704.

Resnick, S. M. & Gibson, D. T. (1996). Regio- and stereospecific oxidation of fluorene, dibenzofuran, and dibenzothiophene by naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816-4. *Appl Environ Microbiol* **62**, 4073-4080.

Reynolds, M. F., Costas, M., Ito, M., Jo, D. H., Tipton, A. A., Whiting, A. K. & Que, L., Jr. (2003). 4-nitrocatechol as a probe of a Mn(II)-dependent extradiol-cleaving catechol dioxygenase (MndD): comparison with relevant Fe(II) and Mn(II) model complexes. *J Biol Inorg Chem* **8**, 263-272.

Rogers, J. (1998). Soil bioremediation: review of land treatment and composting options. *Land Contam Reclam* **6**, 215-222.

Romine, M. F., Fredrickson, J. K. & Li, S. M. (1999a). Induction of aromatic catabolic activity in *Sphingomonas aromaticivorans* strain F199. *J Ind Microbiol Biotechnol* **23**, 303-313.

Romine, M. F., Stillwell, L. C., Wong, K. K., Thurston, S. J., Sisk, E. C., Sensen, C., Gaasterland, T., Fredrickson, J. K. & Saffer, J. D. (1999b). Complete sequence of a 184-kilobase catabolic plasmid from *Sphingomonas aromaticivorans* F199. *J Bacteriol* **181**, 1585-1602.

Rousseaux, S., Hartmann, A., Lagacherie, B., Piutti, S., Andreux, F. & Soulas, G. (2003). Inoculation of an atrazine-degrading strain, *Chelatobacter heintzii* Cit1, in four different soils: effects of different inoculum densities. *Chemosphere* **51**, 569-576.

Safe, S. (1990). Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). *Crit Rev Toxicol* **21**, 51-88.

Sakai, M., Masai, E., Asami, H., Sugiyama, K., Kimbara, K. & Fukuda, M. (2002). Diversity of 2,3-dihydroxybiphenyl dioxygenase genes in a strong PCB degrader, *Rhodococcus* sp. strain RHA1. *J Biosci Bioeng* **93**, 421-427.

Sakurai, H., Shimomura, S. & Yoshimura, T. (1983). Model of the extradiol-cleaving manganese(II) dioxygenase penicillamine-manganese(II) complex. *Biochem Biophys Res Commun* **115**, 618-624.

Salata, G. G., Wade, T. L., Sericano, J. L., Davis, J. W. & Brooks, J. M. (1995). Analysis of Gulf of Mexico bottlenose dolphins for organochlorine pesticides and PCBs. *Environ Pollut* **88**, 167-175.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). Molecular cloning: a laboratory manual. *Second edition Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA*.

Sander, P., Wittich, R. M., Fortnagel, P., Wilkes, H. & Francke, W. (1991). Degradation of 1,2,4-Trichloro- and 1,2,4,5-Tetrachlorobenzene by *Pseudomonas* Strains. *Appl Environ Microbiol* **57**, 1430-1440.

Sanford, R. A., Cole, J. R., Loffler, F. E. & Tiedje, J. M. (1996). Characterization of *Desulfitobacterium chlororespirans* sp. nov., which grows by coupling the oxidation of lactate to the reductive dechlorination of 3-chloro-4-hydroxybenzoate. *Appl Environ Microbiol* **62**, 3800-3808.

Sanvoisin, J., Lanley, G. J. & Bugg, T. D. H. (1995). Mechanism of extradiol catechol dioxygenases: evidence for a lactone intermediate in the 2,3-dihydroxyphenylpropionate 1,2-dioxygenase reaction. *J Am Chem Soc* **117**, 7836 -7837.

Sasaki J., Arey J. & W., H. (1995). Formation of mutagens from the photooxidations of 2-4 ring-PAH. *Environ Sci Technol* **29**, 1324-1335.

Sato, S. I., Nam, J. W., Kasuga, K., Nojiri, H., Yamane, H. & Omori, T. (1997a). Identification and characterization of genes encoding carbazole 1,9a-dioxygenase in *Pseudomonas* sp. strain CA10. *J Bacteriol* **179**, 4850-4858.

Sato, S. I., Ouchiyama, N., Kimura, T., Nojiri, H., Yamane, H. & Omori, T. (1997b). Cloning of genes involved in carbazole degradation of *Pseudomonas* sp. strain CA10: nucleotide sequences of genes and characterization of *meta*-cleavage enzymes and hydrolase. *J Bacteriol* **179,** 4841-4849.

Schindowski, A., Wittich, R.-M. & Fortnagel, P. (1991). Catabolism of 3,5-dichlorosalicylate by *Pseudomonas* species strain JWS. *FEMS Microbiol Lett* **84,** 63–70.

Schmid, A., Rothe, B., Altenbuchner, J., Ludwig, W. & Engesser, K. H. (1997). Characterization of three distinct extradiol dioxygenases involved in mineralization of dibenzofuran by *Terrabacter* sp. strain DPO360. *J Bacteriol* **179,** 53-62.

Schneider, J., Grosser, R. J., Jayasimhulu, K., Xue, W., Kinkle, B. & Warshawsky, D. (2000). Biodegradation of carbazole by *Ralstonia* sp. RJGII.123 isolated from a hydrocarbon contaminated soil. *Can J Microbiol* **46,** 269-277.

Schreiner, G., Wiedmann, T., Schimmel , H. & Ballschmitter, K. (1997). Influence of the substitution pattern on the microbial degradation of mono- to tetrachlorinated dibenzo-*p*-dioxins and dibenzofurans. *Chemosphere* **34,** 1315-1331.

Seeger, M., Timmis, K. N. & Hofer, B. (1995). Degradation of chlorobiphenyls catalyzed by the bph-encoded biphenyl-2,3-dioxygenase and biphenyl-2,3-dihydrodiol-2,3-dehydrogenase of *Pseudomonas* sp. LB400. *FEMS Microbiol Lett* **133,** 259-264.

Seeger, M., Zielinski, M., Timmis, K. N. & Hofer, B. (1999). Regiospecificity of dioxygenation of di- to pentachlorobiphenyls and their degradation to chlorobenzoates by the *bph*-encoded catabolic pathway of *Burkholderia* sp. strain LB400. *Appl Environ Microbiol* **65,** 3614-3621.

Seeger, M., Camara, B. & Hofer, B. (2001). Dehalogenation, denitration, dehydroxylation, and angular attack on substituted biphenyls and related compounds by a biphenyl dioxygenase. *J Bacteriol* **183,** 3548-3555.

Selifonov, S. A., Slepen'kin, A. V., Adanin, V. M., Nefedova, M. & Starovoitov, II (1991). Oxidation of dibenzofuran by *Pseudomonas* strains harboring plasmids of naphthalene degradation. *Mikrobiologiia* **60,** 67-71.

Selifonov, S. A. (1992). Microbial oxidation of adamantanone by *Pseudomonas putida* carrying the camphor catabolic plasmid. *Biochem Biophys Res Commun* **186,** 1429-1436.

Seto, M., Masai, E., Ida, M., Hatta, T., Kimbara, K., Fukuda, M. & Yano, K. (1995). Multiple polychlorinated biphenyl transformation systems in the Gram-positive bacterium *Rhodococcus* sp. strain RHA1. *Appl Environ Microbiol* **61,** 4510-4513.

Sheldrake, G. (1992). Biologically derived arene *cis*-dihydrodiols as synthetic building blocks. In *Chirality in industry*, pp. 127-166. Edited by G. N. S. A. N. Collins, and J. Crosby. Chichester, United Kingdom: John Wiley & Sons Ltd.

Shimizu, S., Kobayashi, H., Masai, E. & Fukuda, M. (2001). Characterization of the 450-kb linear plasmid in a polychlorinated biphenyl degrader, *Rhodococcus* sp. strain RHA1. *Appl Environ Microbiol* **67**, 2021-2028.

Shu, L., Chiou, Y. M., Orville, A. M., Miller, M. A., Lipscomb, J. D. & Que, L., Jr. (1995). X-ray absorption spectroscopic studies of the Fe(II) active site of catechol 2,3-dioxygenase. Implications for the extradiol cleavage mechanism. *Biochemistry* **34**, 6649-6659.

Silva, E., Fialho, A. M., Sa-Correia, I., Burns, R. G. & Shaw, L. J. (2004). Combined bioaugmentation and biostimulation to cleanup soil contaminated with high concentrations of atrazine. *Environ Sci Technol* **38**, 632-637.

Simon, M. J., Osslund, T. D., Saunders, R. & other authors (1993). Sequences of genes encoding naphthalene dioxygenase in *Pseudomonas putida* strains G7 and NCIB 9816-4. *Gene* **127**, 31-37.

Simoni, S., Klinke, S., Zipper, C., Angst, W. & Kohler, H. E. (1996). Enantioselective metabolism of chiral 3-phenylbutyric acid, an intermediate of linear alkylbenzene degradation, by *Rhodococcus rhodochrous* PB1. *Appl Environ Microbiol* **62**, 749-755.

Singh, A., Van Hamme, J. D. & Ward, O. P. (2007). Surfactants in microbiology and biotechnology: Part 2. Application aspects. *Biotechnol Adv* **25**, 99-121.

Sondossi, M., Sylvestre, M. & Ahmad, D. (1992). Effects of chlorobenzoate transformation on the *Pseudomonas testosteroni* biphenyl and chlorobiphenyl degradation pathway. *Appl Environ Microbiol* **58**, 485-495.

Song, R. H., Hua, Z. Z., Li, H. Z. & Chen, J. (2006). Biodegradation of petroleum hydrocarbons by two *Pseudomonas aeruginosa* strains with different uptake modes. *J Environ Sci Health A Tox Hazard Subst Environ Eng* **41**, 733-748.

Spence, E. L., Langley, G. J. & Bugg, T. D. H., 1 (1996). *Cis-trans* isomerization of a cyclopropyl radical trap catalyzed by extradiol catechol dioxygenases: Evidence for a semiquinone intermediate. *J Am Chem Soc* **118**, 8336-8343.

Stackebrandt, E., Rainey, F. A., Ward-Rainey, N. L. & (1997). Proposal for a new hierarchic classification system, *Actinobacteria* classis nov. *Int J Syst Bacteriol* **47**, 479-491.

Strubel, V., Rast, H. G., Fietz, W., Knackmuss, H. J. & Engesser, K. H. (1989). Enrichment of dibenzofuran utilizing bacteria with high co-metabolic potential towards dibenzodioxin and other anellated aromatics. *FEMS Microbiol Lett* **49**, 233-238.

Strubel, V., Engesser, K. H., Fischer, P. & Knackmuss, H. J. (1991). 3-(2-hydroxyphenyl)catechol as substrate for proximal *meta* ring cleavage in dibenzofuran degradation by *Brevibacterium* sp. strain DPO 1361. *J Bacteriol* **173**, 1932-1937.

Studier, F. W. & Moffatt, B. A. (1986). Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J Mol Biol* **189**, 113-130.

Subramanian, V., Liu, T. N., Yeh, W. K. & Gibson, D. T. (1979). Toluene dioxygenase: purification of an iron-sulfur protein by affinity chromatography. *Biochem Biophys Res Commun* **91**, 1131-1139.

Suenaga., H., Ohnuki., T. & Miyazaki., K. (2007). Functional screening of a metagenomic library forgenes involved in microbial degradation of aromatic compounds. *Environ Microbiol* **9**, 2289–2297.

Sugimoto, K., Senda, T., Aoshima, H., Masai, E., Fukuda, M. & Mitsui, Y. (1999). Crystal structure of an aromatic ring opening dioxygenase LigAB, a protocatechuate 4,5-dioxygenase, under aerobic conditions. *Structure* **7**, 953-965.

Taguchi, K., Motoyama, M. & Kudo, T. (2004). Multiplicity of 2,3-dihydroxybiphenyl dioxygenase genes in the Gram-positive polychlorinated biphenyl degrading bacterium *Rhodococcus rhodochrous* K37. *Biosci Biotechnol Biochem* **68**, 787-795.

Takagi, T., Habe, H., Yoshida, T., Yamane, H., Omori, T. & Nojiri, H. (2005). Characterization of [3Fe-4S] ferredoxin DbfA3, which functions in the angular dioxygenase system of *Terrabacter* sp. strain DBF63. *Appl Microbiol Biotechnol* **68**, 336-345.

Takeda, H., Hara, N., Sakai, M., Yamada, A., Miyauchi, K., Masai, E. & Fukuda, M. (2004a). Biphenyl-inducible promoters in a polychlorinated biphenyl-degrading bacterium, *Rhodococcus* sp. RHA1. *Biosci Biotechnol Biochem* **68**, 1249-1258.

Takeda, H., Yamada, A., Miyauchi, K., Masai, E. & Fukuda, M. (2004b). Characterization of transcriptional regulatory genes for biphenyl degradation in *Rhodococcus* sp. strain RHA1. *J Bacteriol* **186**, 2134-2146.

Trenz, S. P., Engesser, K. H., Fischer, P. & Knackmuss, H. J. (1994). Degradation of fluorene by *Brevibacterium* sp. strain DPO 1361: a novel C-C bond cleavage mechanism via 1,10-dihydro-1,10-dihydroxyfluoren-9-one. *J Bacteriol* **176**, 789-795.

Urata, M., Uchimura, H., Noguchi, H. & other authors (2006). Plasmid pCAR3 contains multiple gene sets involved in the conversion of carbazole to anthranilate. *Appl Environ Microbiol* **72**, 3198-3205.

Vaillancourt, F. H., Han, S., Fortin, P. D., Bolin, J. T. & Eltis, L. D. (1998). Molecular basis for the stabilization and inhibition of 2, 3-dihydroxybiphenyl 1,2-dioxygenase by t-butanol. *J Biol Chem* **273**, 34887-34895.

Vaillancourt, F. H., Labbe, G., Drouin, N. M., Fortin, P. D. & Eltis, L. D. (2002). The mechanism-based inactivation of 2,3-dihydroxybiphenyl 1,2-dioxygenase by catecholic substrates. *J Biol Chem* **277**, 2019-2027.

Vaillancourt, F. H., Haro, M. A., Drouin, N. M., Karim, Z., Maaroufi, H. & Eltis, L. D. (2003). Characterization of extradiol dioxygenases from a polychlorinated biphenyl-degrading strain that possess higher specificities for chlorinated metabolites. *J Bacteriol* **185**, 1253-1260.

Vaillancourt, F. H., Fortin, P. D., Labbe, G., Drouin, N. M., Karim, Z., Agar, N. Y. & Eltis, L. D. (2005). Molecular basis for the substrate selectivity of bicyclic and monocyclic extradiol dioxygenases. *Biochem Biophys Res Commun* **338**, 215-222.

Vaillancourt, F. H., Bolin, J. T. & Eltis, L. D. (2006). The ins and outs of ring-cleaving dioxygenases. *Crit Rev Biochem Mol Biol* **41**, 241-267.

Vetting, M. W., Wackett, L. P., Que, L., Jr., Lipscomb, J. D. & Ohlendorf, D. H. (2004). Crystallographic comparison of manganese- and iron-dependent homoprotocatechuate 2,3-dioxygenases. *J Bacteriol* **186**, 1945-1958.

Wang, Y., Garnon, J., Labbe, D., Bergeron, H. & Lau, P. C. (1995). Sequence and expression of the *bpdC1C2BADE* genes involved in the initial steps of biphenyl/chlorobiphenyl degradation by *Rhodococcus* sp. M5. *Gene* **164**, 117-122.

Wang, Y., Yamazoe, A., Suzuki, S., Liu, C. T., Aono, T. & Oyaizu, H. (2004). Isolation and characterization of dibenzofuran-degrading *Comamonas* sp. strains isolated from white clover roots. *Curr Microbiol* **49**, 288-294.

Warren, R., Hsiao, W. W., Kudo, H. & other authors (2004). Functional characterization of a catabolic plasmid from polychlorinated- biphenyl-degrading *Rhodococcus* sp. strain RHA1. *J Bacteriol* **186**, 7783-7795.

Wenk, M., Baumgartner, T., Dobovsek, J., Fuchs, T., Kucsera, J., Zopfi, J. & Stucki, G. (1998). Rapid atrazine mineralisation in soil slurry and moist soil by inoculation of an atrazine-degrading *Pseudomonas* sp. strain. *Appl Microbiol Biotechnol* **49**, 624-630.

Werlen, C., Kohler, H. P. & van der Meer, J. R. (1996). The broad substrate chlorobenzene dioxygenase and cis-chlorobenzene dihydrodiol dehydrogenase of *Pseudomonas* sp. strain P51 are linked evolutionarily to the enzymes for benzene and toluene degradation. *J Biol Chem* **271**, 4009-4016.

Whiting, A. K., Boldt, Y. R., Hendrich, M. P., Wackett, L. P. & Que, L., Jr. (1996). Manganese(II)-dependent extradiol-cleaving catechol dioxygenase from *Arthrobacter globiformis* CM-2. *Biochemistry* **35**, 160-170.

Widada, J., Nojiri, H., Yoshida, T., Habe, H. & Omori, T. (2002). Enhanced degradation of carbazole and 2,3-dichlorodibenzo-*p*-dioxin in soils by *Pseudomonas resinovorans* strain CA10. *Chemosphere* **49**, 485-491.

Wiegel, J. & Wu, Q. (2000). Microbial reductive dehalogenation of polychlorinated biphenyls. *FEMS Microbiol Ecol* **32**, 1-15.

Wilkes, H., Wittich, R., Timmis, K. N., Fortnagel, P. & Francke, W. (1996). Degradation of chlorinated dibenzofurans and dibenzo-*p*-dioxins by *Sphingomonas* sp. strain RW1. *Appl Environ Microbiol* **62**, 367-371.

Wittich, R.-M., Schmidt, S. & Fortnagel, P. (1990). Bacterial degradation of 3- and 4-carboxybiphenyl ether by *Pseudomonas* sp. NSS2. *FEMS Microbiol Lett* **67,** 157-160.

Wittich, R. M., Wilkes, H., Sinnwell, V., Francke, W. & Fortnagel, P. (1992). Metabolism of dibenzo-*p*-dioxin by *Sphingomonas* sp. strain RW1. *Appl Environ Microbiol* **58,** 1005-1010.

Wittich, R. M. (1998). Degradation of dioxin-like compounds by microorganisms. *Appl Microbiol Biotechnol* **49,** 489-499.

Wittich, R. M., Strompl, C., Moore, E. R., Blasco, R. & Timmis, K. N. (1999). Interaction of Sphingomonas and *Pseudomonas* strains in the degradation of chlorinated dibenzofurans. *J Ind Microbiol Biotechnol* **23,** 353-358.

Witzig, R., Junca, H., Hecht, H. J. & Pieper, D. H. (2006). Assessment of toluene/biphenyl dioxygenase gene diversity in benzene-polluted soils: links between benzene biodegradation and genes similar to those encoding isopropylbenzene dioxygenases. *Appl Environ Microbiol* **72,** 3504-3514.

Wolgel, S. A., Dege, J. E., Perkins-Olson, P. E., Jaurez-Garcia, C. H., Crawford, R. L., Munck, E. & Lipscomb, J. D. (1993). Purification and characterization of protocatechuate 2,3-dioxygenase from *Bacillus macerans*: a new extradiol catecholic dioxygenase. *J Bacteriol* **175,** 4414-4426.

Wu, Q., Watts, J. E., Sowers, K. R. & May, H. D. (2002). Identification of a bacterium that specifically catalyzes the reductive dechlorination of polychlorinated biphenyls with doubly flanked chlorines. *Appl Environ Microbiol* **68,** 807-812.

Yabuuchi, E. (2001). Current topics on classification and nomenclature of bacteria. 7. Taxonomic outline of Archeae and Bacteria in the Second Edition of Bergey's Manual of Systematic Bacteriology. *Kansenshogaku Zasshi* **75,** 653-655.

Yamazoe, A., Yagi, O. & Oyaizu, H. (2004a). Degradation of polycyclic aromatic hydrocarbons by a newly isolated dibenzofuran-utilizing *Janibacter* sp. strain YY-1. *Appl Microbiol Biotechnol* **65,** 211-218.

Yamazoe, A., Yagi, O. & Oyaizu, H. (2004b). Biotransformation of fluorene, diphenyl ether, dibenzo-*p*-dioxin and carbazole by *Janibacter* sp. *Biotechnol Lett* **26,** 479-486.

Yates, J. R. & Mondello, F. J. (1989). Sequence similarities in the genes encoding polychlorinated biphenyl degradation by *Pseudomonas* strain LB400 and *Alcaligenes eutrophus* H850. *J Bacteriol* **171,** 1733-1735.

Yeh, W. K., Gibson, D. T. & Liu, T. N. (1977). Toluene dioxygenase: a multicomponent enzyme system. *Biochem Biophys Res Commun* **78,** 401-410.

Yoon, J. H., Lee, K. C., Kang, S. S., Kho, Y. H., Kang, K. H. & Park, Y. H. (2000). *Janibacter terrae* sp. nov., a bacterium isolated from soil around a waste water treatment plant. *Int J Syst Evol Microbiol* **50,** 1821-1827.

Yu, H., Zhu, L. & Zhou, W. (2007). Enhanced desorption and biodegradation of phenanthrene in soil-water systems with the presence of anionic-nonionic mixed surfactants. *J Hazard Mater* **142**, 354-361.

Zielinski, M., Backhaus, S. & Hofer, B. (2002). The principal determinants for the structure of the substrate-binding pocket are located within a central core of a biphenyl dioxygenase alpha subunit. *Microbiology* **148**, 2439-2448.

Zylstra, G. J., Kim, E. & Goyal, A. K. (1997). Comparative molecular analysis of genes for polycyclic aromatic hydrocarbon degradation. *Genet Eng* **19**, 257-269.

A. APENDIX

A1: The 16S rRNA gene sequence of *Rhodococcus* sp. strain HA01.

TAGAGTTTTTATCATGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAAGCCCAGCTTGC
TGGGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGGTGATCTGCCCTGCACTCTGGGATAAGCCTGGGAAACTGGGT
CTAATACCGGATATGACCTCNNGATGCATGTTGnGGGGTGGAAAGTTTTTCGGTGCAGGATGAGCCCGCGGCCTATCAG
CTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAG
ACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACTGAATGGGTAGAAGCGCAAGCCTGATGCAGCGA
CGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCACCCATGACGAAGCGCAAGTGACGGTAGTGGGAGAA
GAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTACTGGGCGTAAAG
AGCTCGTAGGCGGTTTGTCGCGTCGTCTGTGAAATCCCGCAGCTCAACTGCGGGCTTGCAGGCGATACGGGCAGACTCG
AGTACTGCAGGGGAGACTGGAATTCCTGGTGTAGCGGTTGAAATGGCGGCAGATATCAGGAGGAACACCGGTGGCGAAG
GCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCACG
CCGTAAACGGTGGGCGCTAGGTGTGGGTTTCCTTCCACGGGATCCGTGCCGTAGCCAACGCATTAAGCGCCCCGCCTGG
GGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGATTAATTCGAT
GCAACGCGAAGAAACCTTACCTGGGTTTGACATGTACCGGACGACTGCAGAGATGTGGTTTCCCTTGTGGCCGGTAGAC
AGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTGTG
TTGCCAGCACGTGATGGTGGGGACTCGCAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCAT
CATGCCCCTTATGTCCAGGGCTTCACACATGCTACAATGGTCGGTACAGAGGGCTGCGATACCGTGAGGTGGAGCGAAT
CCCTTAAAGCCGATCTCAGTTCGGATCGGGGTCTGCAACTCGACCCCGTGAAGTCGGAGTCGCTAGTAATCGCAGATCA
GCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCATGAAAGTCGGTAACACCCGAAGCCG
GTGGCCTAACCCCTTGTGGGAGGGAGCCGTCGAAGGTGGGATCGGCGATTGGGACGAAGTCGTAACAAGGTAACCCGTA
A

A2. Partial 16S rRNA gene sequence of *Paenibacillus* sp. strain HA01. Sequence corresponds to positions 530 to 1087 according to *E. coli* ribosomal gene.

CGTGCCAGCAGCCGGGGTAATACGTAGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGCCGC
TTAAGTTTGGTGTTTAAGCCCGGGGCTCAACCCCGGATCGCACCGAAAACTGGGTGGCTTGAGTGCAGGAGAGGAAAGC
GGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTTTCTGGACTGTAACT
GACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGG
TGTTAGGGGTTTCGATACCCTTGGTGCCGAAGTCAACACAATAAGCACTCCGCCTGGGGAGTACGCTCGCAAGAGTGAA
ACTCAAAGGAATTGACGGGGACCCGCACAAGCAGTGGAGTATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAG
G*T*CTTGACATCCCTCTGACCGGTACAGAGATGTACCTTTCCTTCGGGACAGAGGAGACAGGTGGTGCATGGTTGTCGTC
AGCTCGTGTCGTGAGATGTTGCCTTAA

A3: Sequence of a 4,421 bp fragment comprising *dfdA1A2A3A4* of *Rhodococcus* sp. strain HA01.

GGGGGGATATTTGGCCTCACCCGAGGGAGGATTTTCACGACGGCTGCGGTGTGATGTAACACTCACCGCACCAGCCCTC
GAACCCGTGAGACGGACTTGATGACGGGACGGAGTTCGGCGGTGGTGTACAGGACGACCAGGTAACCGGGAAGTCCTCT
ACAGCGCTGACCCCAAGAAATGCAGCGCGTTCGAATCCCCCCGACAAACGAAGTCGCAACTGAAGCTAGGAATGGAGGC
AACAATGCTGACTGTGAATGACAGTGGTCAACTGGTGAGCCCGAACGGGCAGACACCTCAGGCACCACCTTTGAATCCC
GCCCTGTCGTCTCTGCTCAAGGAACTGTCCGAGAGCGAGGGTGGCCTGCTGGACCGGCGCATGTTTTTCGACCCTGAGA
TCTACAAGGTTGAACTTGAGCGCGTCTTTGCACGATCATGGTCCTTTCTCTGCCATGAAAGCCAGCTGGCCAAGGCCGG
GGACTTCTTCTCGACCTACATCGGCGCCGATCCCGTCGTGGTGACCCGACAGCGCGACGGATCGATCAGCGCGGTGCTC
AACTCTTGTCGCCATCGTGGGATGAAGGTCTGCCGCGCCGACTGGGGGAACGCGAAGGCCTTCACCTGCACGTACCACG
GTTGGTCGTACAGCACGGATGGCTCGTTGGTGAGCGTGCCCCGCGAGGAATACGCCTACTACAACGAGATCGACAAGTC
GAAGTTGGGATTGCTGCGGGTTCCACAGGTGCAGTCCTACAAAGGGCTGGTTTTCGGTTGCTTCGATCCCGAAGCGCCG

TCGCTTGTCGACTTCTTGGGCGACATGACCTACTACTTGGACATCCTGCTTGACCGTGTGGATGGCGGCACCGAAGTCA
TCTCCGGTGTCCACAAGTGGAAGATGCGGGGCAACTGGAAGCTTGCCGCCGAGCAGTTCAGTGGAGACAACTACCACAC
CATCTCCAGCCATATATCGGTGCTGCTGTCTGAGTTCCCGCCCGAGGCGGCGGACGCCTTCGTGAATATCGACGGGCTC
GAGATCAACCCAGCGGAAGGCCATGGTATTGGTGTTATGTACTCGCCGACCGGAGCGCCGTTCTCGGCGGGGAGCAGCG
AGGCGATCCTGCGCTGGCGCGACGAGACGCGCCAGGAGTCCATCAACCGCCTTGGTAAGGAGCGCGTAGAGGGGATGTC
CTGGACGCACGCCAACGTGTTCCCCAACTTCTCTTACCTCCACGACAGCTCGGTCCTGCGCGTTTGGATGCCCAAGAGT
CCCACCGAGATGGAGGCCTGGTCGTGGTGCATCGTCGACAAGAAGGCTCCGCAGGAGGTGAAGAATGCTTGGCGCACGC
AGGCCATCCGACACTTCAGCCCCGGTGGTACTTGGGAACAGGACGACGGCGAGAACTGGAGTTACTGCTCAGGTGCTGG
GGGTCAGGAGGGAGTGGTGACCCGACTCTCCAAGTTGCATGTCGAGATGGGAGTGGGACACGAGCGCTCTCATCCGACG
CTGCCCGGCAAGGTCAGTCACACCTACAGTGAGCAGAACCAGCGCAGTCTGTACCGACGCTGGGCCGAGTTCATGGCGG
CGGAGTCTTGGAAGGACATCTCCGTGCCGGTGCGTACGACCGAGGTAATCGACCGAAGCGACATGGCGAAGGCGGGAGA
ATCCTGATGAGCGTTCTTGAGAATACGAATACAGAGGTTATTGACGTCGCCCGTGCGGTCGAGAAGTTCTACTACAAGG
AGGCGCGACTCCTTGACGACAGGCTCTTCACGGAGTGGCTCACATTGTGGGCCGACGATGCCCACCTGTGGGCGCCTCT
CCGGTATAACCTGTCTCGGCGGGAGCAGCAGTTCGAGTATTCCGGTGAAGACGACTTCGGATACTTCGACGACGACAAA
CCGAATCTCGAGAAGCGGGTGCGGGGGTTGGAGACCGGGCAGGCGTGGGCCGAGGATCCCCCGACGCGCACCAGGCGCC
TCATTACGAACGTCGAAGTGGAGTCGGACGATTCCGGTGTAGGAGACTACCGGGCCCGGTCCCACTTCCTCGTCTATCG
CAACCGCATGGAAGCCGATGTTGACCTGCACGCTGGATGTCGGCGCGACATCCTCCGCCGGACTGCCACGGACGGTCTG
CTCATCGCCCGCCGCGAGGTCATCCTAGACAACAACGTGTTGCTGTCTAGGAATCTGAGCATCTTCTTCTGAGAAGCGA
GGTCACAGTGACGTTGAATCACACGCAGATAGCCCGCTGCGCGCGGTCAAGTATCAGCAGTTGGCTTCGGGCACGATGA
CCAACAACGACGTGGAAGTGGCGCTGCCGAACGTCGAGGGCCGCACATGGCGCCGTGCCTGTGCGGCCCACGACGTGCC
CGAGGACGAAGGTCTGTGCCTCGGTACGCTGCCGCCCGTCTCGGTGTTTTTAACAGAGGGCGAGTACTTCTGTATCGAC
GATACGTGCACCCACGAGACCTACTCGTTGGCGGACGGGTGGGTCGCGGACGGTTTCGTCGAATGCGCCCTCCACCTCG
CTAAGTTCAACTTGCGCACCGGCGAGCCGCTCGCGCCGCCCGCCACGACGGCTGTGGCCGTCCATCCCGTCGCACTCGT
CGACGGGGTCCTTTATGTTGCGCTTCCGAGCACGTACCTCATCAAGGAGTGAGAGCCGTGGGTTGGCTTCAGGACTACT
CGGTCATCGTCACCGGAGCCGGTTCCGGTCTCGGTCGCGCGACATCGTCGAGCGGTATGTCGAGGAGGGCGCTCGCGTC
GTCGCCTTCGACCGCTCCGAGGTGAAGCCACACCGACAGAGAGTCAGCCCACAGTTCATTCTAGGTGGCAGCGACGGGT
CGGCGAGGAGAGTCATTCTAGGTGGCAGCGACGGGTCGGCGAGGAGAGGCTGGATGTCGGGCGGTTACTGACTGGCGCT
CCATGGCTCGGGACAGCCCGGGGGCGGCGGTGTTTTGGGAGGCCCTTCGCGCGCGACCCCCACCGGCCATCACACCGAG
CCTCACCGAGTCCGAACAACAACCATCATCTCGATCCCCGCCAGGAAGAGGGTGCACCAATGACCGCACCGCACCACGT
CATCGTCGGTGGCAGTGCTGCCGGTGTCGCAGCGGCACTAGCCATGCGGAGAAATGGCTTCGAGGGTCGGATCACTCTC
GTGGAAGCAGCCTCCGAGGAGCCCTACGAGCGACCGCCTCTGTCGAAGTCTTTCACCGACCTTGACGCGCCGCGTCGGA
TCCTCCCACCGAGCACGTACGTCGAGGAAGACATCGACCTGCTGCTCGGCATGCCGGTCGCAGCGCTCGATGTCGACCG
GAAGGTGGTGCGGTTGCCTGACGGCGAGGGACTCGGGGCGGATGCCGTGCTAGTGGCGACCGGTGTCAACGCTCGGCGT
CTGGGAGTTCCGGGAGAATATCTCGAGCATGTCCTGGTGCTGCGTGGCCTGGCGGATGCACGTGCGCTGGCGGCGCGCC
TCGACGTGGGCGGTCCTTGGGTGATCGTCGGAGGAGGGTTCATCGGCCTCGAGGCGGCGGCCGTCGCGCGGGGAAGAGG
GATCGATGTCACGGTAGTCGAGGCGATGCCGGTGCCGCTGGCCGGCGTGCTGGGCCCTGCCCTTGCAGCCCACGTCCAG
CGGATGCACGAGCGTGAGGGGGTGCGGATTCTGGGGGGGCGCACTGTGACCGAGTTCGTGGGGGAGAGGGAGGTCGAGA
AGGTCGTCCTGGACGATGGCTCGGTTCTGGATGCGGCCACCGTACTCGTTGGCTGCGGGGTGGAGCCCAACGACGAGCT
GGCCCGAGACGCAGGGGTGTACTGCAACGGCGGCATCGTCGCGGACCGTCACGGTCGCACGAGTGTCCCCTGGATCTGG
GCGGCCGGCGACGTCGCCACCTTCGTCAGTCCGTTCACCGGGCGTCGCCAGCGCATCGAGCACTGGGACGTCGCCAATC
GTCTAGGCACAGTCACCGGAGCCAACATGGTTGGGGTACCGGCAGTCAACACAGATGCGCCGTACTTCTGGTCCGATCA
ATACGGACATCGGCTCCAGATGTATGGCCGACACCAGCCAGGCGACCAGTTCGTCGTCCGACCTGGCGTGACCACGGCG
CAGTTCGTCGCATTCTGGGTCCGCGATGGGCGGGTCACCGCGGCGGCTGCGATCGACTCGCCGAAGGAGTTGCGGGCGA
CCAAGCCACTGATCGAGGGACGAGTTCCCGTTATGGCATCGGACCTGATCGACCCGGCCGTCTCATTGCGTGCGCTCGG
GCGTGTCGCTCATCACGAAGCCAGATCGTGATGGCGCATAGGACGATTCCTCCGCTGCCGTCCGTACCCGGGGAGTCGA
CGTCGTGTCGGGCGGCAACGTTCTCCGCATGCCGCAGGAACGAAGAGTAGACGCCGGCGTACCCGAGGGTGAGCGT

A4: Amino acid sequence of DfdA1 from *Rhodococcus* sp. strain HA01. Amino acids differing from the DfdA1 protein of *Terrabacter* sp. strain YK3 (BAC06602) are shown in bold.

MLTVNDSGQLVSPNGQTPQAPP**L**NPALSS**L**LKELSESEGGLLDRRMFFDPEIYKVELERVFARSWSFLCHESQLAKAGD
FFSTYIGADPVVVTRQRDGSISAVLNSCRHRGMKVCRADWGNAKAFTCTYHGWSYSTDGSLVSVPREEYAYYNEIDKSK
LGLLRVPQVQSYKGLVFGCFDPEAPSLVDFLGDMTYYLDILLDRVDGGTEVISGVHKWKMRGNWKLAAEQFSGDNYHTI
SSHISVLLSEFPPEAADAFVNIDGLEINPAEGHGIGVMYSPTGAPFSAGSSEAILRWRDETRQESINRLGKERVEGMSW
THANVFPNFSYLHDSSVLRVWMPKSPTEMEAWSWCIVDKKAPQEVKNAWRTQAIRHFSPGGTWEQDDGENWSYCSGAGG
QEGVVTRLSKLHVEMGVGHERSHPTLPGKVSHTYSEQNQRSLYRRWAEFMAAESWKDISVPVRTTEVIDRSDMAKAGES

A5: Amino acid sequence of DfdA2 from *Rhodococcus* sp. strain HA01.

MSVLENTNTEVIDVARAVEKFYYKEARLLDDRLFTEWLTLWADDAHLWAPLRYNLSRREQQFEYSGEDDFGYFDDDKPN
LEKRVRGLETGQAWAEDPPTRTRRLITNVEVESDDSGVGDYRARSHFLVYRNRMEADVDLHAGCRRDILRRTATDGLLI
ARREVILDNNVLLSRNLSIFF

A6: Amino acid sequence of DfdA3 from *Rhodococcus* sp. strain HA01. Amino acids differing from the DfdA3 protein of *Terrabacter* sp. strain YK3 (BAC06604) are shown in bold.

MTNNDVEVALPNVEGRTWRRACAAHDVPEDEGLC**L**GTLPPVSVF**L**TEGEYFCIDDTCTHETYSLADGWVADGFVECALH
LAKFNLRTGEPLAPPATTAVAVHPVALVDGVLYVALPS**T**YLIKE

A7: Amino acid sequence of DfdA4 from *Rhodococcus* sp. strain HA01. Amino acids differing from the DfdA4 protein of *Terrabacter* sp. strain YK3 (BAC06605) are shown in bold.

MTAPHHVIVGGSAAGVAAALAMRRNGFEGRITLVEAASEEPYERPPLSKSFTDLDAPRRILPPSTYVEEDIDLLLGMPV
AALDVDRKVVRLPDGEGLGADAVLVATGVNARRLGVPGEYLEHVLVLRGLADARALAARLDVGGPWVIVGGGFIGLEAA
AVARGRGIDVTVVEAMPVPLAGVLGPALAAHVQRMHEREGVRILGGRTVTEFVGEREVEKVVLDDGSVLDAATVLVGCG
VEPNDELARDAGVYCNGGIVADRHGRTSVPWIWAAGDVATFVSPFTGRRQRIEHWDVANRLGTVTGANMVGVPAVNTDA
PYFWSDQYGHRLQMYGRHQPGDQFVVRPGVTTAQFVAFWVRDGRVTAAAAIDSPKELRATKPLIEGRVPVMASDLIDPA
VSLRALGRVAH**HEARS**

A8: Sequence of a 4,862 bp fragment comprising *dbfA1A2* of *Rhodococcus* sp. strain HA01.

GAGGAGATCTACAGCCGGCTGGAAGCCGCGACCCCGTCATACGAGCGGGCCGGCGTGGCCCACCTCCCCTTCGACGCGC
AGGGCGCCCTGTACGCCGCGGGCATGGCAAACCTCGGGGCCCAGAACAGTTGGACCAGCAATGACAGCGCTCAGTGATA
CCGCAGACGCCCAGACGATCCGCTTACAGGTGGGCCGTTCGGGTCCGCCCTCCCATCTCACCTCACGCAACCCGTCAGA
CCCCGAGAGGACCCGCTCATGACCAGCATTAGCGAACGCCCCGTTGACGTGGCCGCCGCAGGACTGCACGACCTGGTGA
AGCCCGACGAGGGTTCCGTCAGCCGAACGGTCTTCGTCGACGAGGCAATCTACCGCAAGGAATTGGACCGGGTATTCAC
CAAGACGTGGTTGTTCATCGGCCACGAGTCCCAGTTGAGTGAGCCGGGCGACTACCTGACGAACTTCATGGGCGAGGAC
CCTGTGATCGCCACTCGCGGTGCGGACGGAGTGATCCGGGTGATGCTCAACTCCTGCGCCCACCGGGGCATGGCCGTGT
GTAGCACCGACGCCGGGTCCTCGAAGTTCTTCCGCTGCCCCTACCACGGCTGGACCTACAGCAACAACGGCGATCTGAT
CGGTGTACCGCGCGCAGATACCGTCTACCATGGCGAGCTGGACAAGTCGCGGCTAGGTCTGAAGGCCGTTCCGCGGGTG
GAGAACTACAAGGGTTTCATCTTCGCCAATTGGGACGAGGACGCCATCCCGCTGGTGGACTACCTCGGCGCTGACCAGC
TCTGGTATCTGGACCTGGCCTTCGAGGCGCCGCTCGGCGGGCTCGAGGTGATCGGCCCCACGATGAAGTTCCGGATCAA
GGCCAACTGGAAGCTGGCGGCGGAGAACTTCGCCGGCGACGACTACCACGTGCTCTACACACATGGGTCGGCCTTCCAG
ATCGGCTTCCTCCCCGATTACGACACGCTCGGCGACTACATCGCATACTTCGGCCACGGCCACGGGATGGGCGACATCA
GCAAGCCCGGCCGGGCCTATCAGAACGACGTCGGGATGGCTCAGTTCCTCGGGCCGGAGGCGATCGAGTACGTCAACGC
CGTGCACGAGCGGCTCAAGGCCCGGGTCTCCCCGCTGCAGGCGGAGATGCACGGGCTCGGTCAGGGCAATATCTTCCCG

AACCTGTCATGGATCAAGTTCGGCGTCTTCCACGTCTTCGGGCTCTTCCAATGGCACCCGAGGGGACCGGGTGAGATCG
AGGTCTGGCAGACGGCGCTCTTCGACCGCGACGCGCCGCAGTCGGTCAAGGACTTCGCCCGCACCCAGATGTCCCAGGA
GAACGCCGCGGCCGGGATCTTTGGCCAGGACGATGGCGAGAACTTCGAGCAAATCACCGAGTCCGCCCGCGGGGTGGTC
TCCCAGACCCGGGATTTCAACTACGCGATGGGCCTGGGGCACGAGGGCGAGATCCACGAGGAGGGATACCCCGGCCATT
TGGGGCCCCACTATTCGGAGCAGAACCACCGCAACTTCTATCGCTACTGGCTCGAACTCATGACCACCCCGGGAGAGCA
GAAATGACCAATTCCCTCACCGATCTGCGCCGCGACGTCGAGGACTTCCTCTACAGCGAGGCCAAGATGCTCGACGAGC
AACGCTACGACGAGTGGCTCGACCTGTTCACCGAGGATGTCCATTACTGGATGCCGATCACGGAGACCCGTGAGGTGCG
GCAGCACCGGGACCACGTCCCCGGCGAGTGGTCGCTCATGGAGGAGGACGCCCGTTTCCTCGCCAAGCGGATGGAGCGC
TTGGCCGGTGGCCTTGCCCACTCGGAGCAGCCGCGGTCGCGGACGCGTCGTTTCATCAGCAACGTCCTGGTCACCCCCG
GGCCGGACGGCGACCTGGTAGCGGAGTGCAACTTCATCGTCTTCCAGTCCCGGCGAGCCAACTCCGAGCAGTTCTTCGT
CGGCTCCCGTCGCGACCGGATCGTGACCTCCGGTGAGAGCTGGAAGATCGCCGAACGGACCGTGCTCTTCGACCACCGG
GTGTTGCCCCGTGCCATCTCCATCTTCTTCTGAGGCCCAGGCGGCTGACCTCGACGGCAGTCGGGATGTCGGGGCTGAT
GGCGCACCTGAGCCGAGTTCTTGGCTGCGCATCGGCTCTGCCGACGGCGAGCTCGTAGTCAGCAACGAGTTCGCCCGGG
TGCGGGTACGCGCCGACCGAGACGGCAACGACCTTCGCCTGGCCATCCGTAGCCTGCGCACCGGCCGCGAGGTCTTCCT
TGACGCGCTGCAGCTGGAGAGCCTCACCTGGCTCGACGAGAGGGCCTACACGACATTGCTCTCCGAGCCGTTCGGACCG
GAGTGACCGACCATCCCGCACGACCGAACAGAGAGGAGCCTCAACGTGACGAGGACTGAAGGGGGAATGGCCCAGCCCC
AGGGGAACTCGTTCTGGGTGGACCTGCTGGGAACCGAGGTCCGGTACCGCCAGGCGAGCTCTTACCGCACCCGCAGCAT
CGAGGCAGGCGCCGGTGAGCCGGTAGTCCTGCTGCACGGCGTGAGCGGGCACGCCGAGACCTGGGTGCGCAACATCGCC
GTCCTCGGCCGGGACTTCCGCGTGCATGCCATTGACATGCTGGGTCACGGCTTCACCGACAAGCCGCAGATCGAGTACT
CGATCCGCGCGCTCGCCGAGCACGTGCTCGGCTTCCTCGACGAGATCGGCGCCTCGCGAGCCCACCTGGTCGGGCAGTC
TCTCGGTGGCTGGGTCGCGGCGTTTCTCGCCGTCCACCACCCCGAGCGGGTCGCCTCGCTCGTCAGTGTCACCGGGGCG
GGGCTGCAGGTGGACGCCGACGGTGCCACGCTGACCGAGAACGTCGGCCGGCAGGTCGCGGAGGCTACCACCAAGGCTC
TGGACACCCCGACCCGGGAGAAGGTCCGGACTCGGCTGGAATGGCTGGTCCATGACCCGTCCGTGGTCACCGACGAACT
GGTGGAGACCCGGTACCGGATCTACGCCAGCCCGGACTTCGCCGCCACCGCCGGTGACATGGTCGCCGCATTCACCTCC
CGGCCCCGGCCGGAGGAACTGCTGACCGCCGAGCGGCTCGCGACGATCAACTGCCCGACGCTCGTGCTGTGGACCCGGC
AGAACCCGACCATGCCGTGGCCGGTGGGGGAGGCGGCCAGCCGGATCATTCCCGATGCAACGTTCCGTCTGATGGAGGA
CGCTGGTCACTGGCCGCAGTTCGAGAAGCCGGCGGAATTCAACGCTGTCGTCGGCGGGTTCGTGCGGTCGGTCACCGCG
GGGCGCCGAGGTGGGCGCACCGACGGCGACGGCGGCCCCACGGATCCGGCCACCAACCGGCAACGGGCGGCCCGCTGAT
GGGCAGGCTGGTAGGTGCGTACGCGACGTCCCACACGGCGATGATGATCCGAAAGCTCCAACCGGACAACGACGTGCAC
GCCGCAGTCCATCAGGCCTTCGCTCAGGTGCGTGCGGAAATTGACCGGCTGTCCCCTGACGTGCTCGTCGTCGTCAGCA
GCGAGCACCTAGCATCCTTCAGCTACGACAGCTTTCCGCAGATATGCGTCGGAATCGGAGAGATAGCCACCGGTTGGGG
CGATGGAGGAGTGGCCAGCGCCGAGGTGCCACTTGCTGGCGCCTTCGCTGCCCAGCTGCTCTCCGAAGGGGTGGCCGCC
GGGTTCGACCTCGCGTTCTCCGCCAACCCGAAGATCGACCATGCGTTCATGGCACCATTGACCCTGATCCGACCAGAGA
TGGACATCCCGGTGGTCCCGGTGTTCCAGAACGCCAACACCGAGCCTCTGCCACCCCTGTGGCGCAGCGCCCAACTGGG
GGAACTGCTGCGCGACGTCATTACGCGGCGGCCCGCAGCCGAAACAGTGGTCGTGCTCGGGACCGGCGGGCTCTCCCAC
TGGGTCGGCACGCCGGAGATGGGCCAGATCAACTCCGCTTTCGACGAGCGGTTCCTGCAGCACGTGCGCGCAGGGGACC
TCGGCGCGATCCTCGCCATGAAGACCGCCGACGTCCTCGCTGAGGCCGGCAATGGTGCCCCGGAGATCTGCAACTGGGT
CACCGCGATGGCCGCCGCGTCCAGTCAAAAGGGCTGGGGAGTGTCGGAGCCTGAGCGGACGGCCCGTGGGCCGAGAGGG
GTCGTCCTGGCATACGAGTCCGTCCCGGACTGGGCGACCGGGATCGCGCTGGCTCGGTTGGCCCCCGAGGAGGAACCGT
GAACTTACCCCTGGACCAGGTCATCCGCCGGGTCGTCCGGGATCCGGAGTTTCGTTCCATTGCGGAAGAGTCGGGCCAG
CTCGCGGCCGACCTCGCAGGCGTACGGCTGGCCGATCTTGCGGCCGTCCTGGAGGGCGACCTGGTCACCCTCCAGCAAA
GGGGCGCCCACCCACTGCTGATCATGCAGCTGGCCGGCGCGCTCAGGATCGATCCGATGCGGCGCTTCGCCGCCGAACA
GACTGCCCATGACTTAACCACGGAGGGACGATGAACATGATGAACATTACGGTGGACCTCGAGCGGTGTGAGGGCTTCG
CCTCGTGCGTTGTGACCGCGCCCGAGCTCTTCGACCTCGATGACGAGCGAAGCGTCGCCGTCGTTCTGGAGCCTGTTGA
CGGTGGCCCCCGCACCCGCGCCTTAGCGCTCGAGGCCGCCGCGAGCTGCCCGGTGCGGGCCATCACCGTCTCCGACCCC
CTGGTGGACGAACCATCGCTGCCGCGGCGCGGCCGGTGACCGACGGAGTTTCGCCGGACGCGGTGCAACCGCGGTCGAG
AACGAACTCCTCGGGCGACGGGGCCGGACGCCCTTGCCCGGTCG

A9: Amino acid sequence of DbfA1 from *Rhodococcus* sp. strain HA01. Amino acids differing from the DbfA1 protein of *Terrabacter* sp. strain DBF63 (BAC75993) are shown in bold.

MTSISERPVDVA**A**AGLHDLVKPDEG**S**VSRTVFVDEAIYRKELDRVFTKTWLFIGHESQLSEPGDYLTNFMGEDPVIATR
GADGVIRVMLNSCAHRGMAVCSTDAGSSKFFRCPYHGWTYSNNGDLIGVPRADTVYHGELDKSRLGLKAVPRVENYKGF
IFANWDEDAIPLVDYLGADQLWYLDLAFEAPLGGLEVIGPTMKFRIKANWKLAAENFAGDDYHVLYTHGSAFQIGFLPD
YDTLGDYIA**Y**F**G**HGHGMGDI**S**KPGRAYQNDVGMAQFLGPEAIEYVNAVHERLKARVSPLQAEMHGLGQGNIFPNLSWIK
FGVFHVFGLFQWHPRGPGEIEVWQTALFDRDAPQSVKD**F**ARTQMSQENAAAGIFGQDDGENFEQITESARGVVSQTRDF
NYAMGLGHEGEIHEEGYPGHLGPHYSEQNHRNFYRYWLELMTTPGEQK

A10: Amino acid sequence of DbfA2 from *Rhodococcus* sp. strain HA01. Amino acids differing from the DbfA2 protein of *Terrabacter* sp. strain DBF63 (BAC75994) are shown in bold.

MT**N**SLTDLRRDVEDFLYSEAKMLDEQRYDEWLDLFTEDVHYWMPITETREVRQHRDHVPGEWSLM**E**EDARFLAKRMERL
AGGLAHSEQPRSRTRRFISNVLVTPGPDGDLVAECNFI**V**FQSRRANSEQFFVGSRRDRIVTSGE**S**WKIAERTVL**F**DHRV
LPRAISIFF

A11: Deduced amino acid sequence of *meta*-cleavage compound hydrolase from *Rhodococcus* sp. strain HA01. Amino acids differing from the protein of *Rhodococcus* sp. strain YK2 (BAC00805) are shown in bold.

MAQPQGNSFWVDLLGTEVRYRQASSYRTRSIEAGAGEPVVLLHGVSGHAETWVRN**I**AVLGRDFRVHAIDMLGHGFTDKP
QIEYSIRALAEHVLGFLDEIGASRAHLVGQSLGGWVAAFLAVHHPERVASLVSVTGAGLQ**V**DADGATLTENVGRQVAEA
TTKALDTPTREKVRTRLEWLVHDPSVVTDELVETRYRIYASPDFAATAGDMVAAFTSR**P**RPEELLTAERLATINCPTLV
LWTRQNPTMPWPVGEAASRIIPDATFRLMEDAGHWPQFEKPAEF**N**AVVGGFVRSVTAGR**R**GGRTDGDGGPTDPATNRQR
AAR

A12: Deduced amino acid sequence of extradiol dioxygenase from *Rhodococcus* sp. strain HA01. Amino acids differing from the protein of *Rhodococcus* sp. strain YK2 (BAC00806) are shown in bold.

MGRLVGAYATSHTAMMIRKLQPDNDVHAAVHQAFAQVRAEIDRLSPDVLVVVSSEHL**A**SFSYDSFPQICVGIGEIATGW
GDGGVASAEVPLAGAFAAQLLSEGVAAGFDLAFSANPKIDHAFMAPLTLIRPEMDIPVVPVFQNANTEPLPPLWRSAQL
GELLRDVITRRPAAETVVVLGTGGLSHWVGTPEMGQINSAFDERFLQHVRAGDLGAILAMKTADVLAEAGNGAPEICNW
VTAMAAASSQ**K**GWGVSEPERTARGPRGVVLAYESVPDWATGIALARLAPEEEP

A13: Deduced amino acid sequence of a ferredoxin-like protein from *Rhodococcus* sp. strain HA01. Amino acid differing from the protein of *Rhodococcus* sp. strain YK2 (BAC00808) is shown in bold.

MNMMNITVDLERCEGFASCVVTAPELFDLDDERSVAVVLEPVDGGPRTRALALEAAASCPVRA**I**TVSDPLVDEPSLPRR
GR

A14: Deduced amino acid sequence of extradiol dioxygenase Edo4 from *Sphingomonas wittichii* RW1.

MSEISSLGYVGYSVTDLDRWEELAVDILGFVPGRRNPGRSLGLRMDKLEQRIVLERDGKDDLKYVGWLFDTEDDLDGFV
DKARGAGVDIRPQSAEIAKQRAVDRVHAVTDPNGVIHEFAFGPKFASAHEPFLSKVLRGGFVTGRLGVGHVLEVARDYG
ETVAFARRVLGLKVSDYIRGPQPMPNGIFDVEAAFFHTRTGRHHSLATAEVPTPLRIHHMMVEVSDMDDVGLAYDRCRA
AGFPIGMELGHHPNDGMFSFYVRTPSGFLIEFGWGGVVIDDADWEVKTYSQLSDWGHAHAH

A15: Deduced amino acid sequence of extradiol dioxygenase Edo5 from *Sphingomonas wittichii* RW1.

MAVRSLAYIRVETRDLAAWRSFAEGVVGAAATPDSNDERLLLRLDGRPWRFCIEKGEQDRYLCAGLECPDEAQWQATVD
RLAKAGVALERADAATIAQRHVRGMVSLADPAGNRIELIWGNVVAGTPFVSPAGVPAFITGEMGFGHVVVPTNCYEETR
RFYKELLGFGDSDEMRVFFPGGPEQGLGMSFMHATGPRHHSVAVGEFPAPSGLIHTMVEVPSVDDVGLALDRALAAGVH
ISSTLGRHTNDKMISFYMRTPSGFDIEFGCGGEQTADWTTVTPTFTIKEDLWGHKWDFGT

A16: Deduced amino acid sequence of extradiol dioxygenase Edo 6 from *Sphingomonas wittichii* RW1.

MTIRSLGYIGFGAPDPAAWLRYGTDILGLMPARALAGEDWGIPAIPGSGPKSGGSGIAEDGSVYLKMDDWQWRIAVHPD
DTNRGVKYLGLEVEDEADLAAHVARLREAGFVAEMGSAEQAEARSVSGIAHTTDPCGNAIELFHGPTVDRKFQSPLGME
FLAGPLGIGHVNLLTSNRLAAARDFYMRALGFRLADYIAFGGGNSANFYRCNPRHHSIGLLEVGRAIGVHHLMLEVTKV
DMVLQCLERVTDAGIAVTSTVGRHVNDNMLSFYMRSPFGFEVEIGFDGRLLDEDWTPNRFVEGDIWGHRGLDPETIARN
LAAMPQGS

A17: Deduced amino acid sequence of extradiol dioxygenase Edo7 from *Sphingomonas wittichii* RW1.

MRRLVRRGLLRSAHSGCTLRTAGRPPPDICGPDKANIWSGSDVQLSYVGLGVADVPAWLSFATDVLGLTAEREADEARL
RVDGKAWRIAVRPSPADDLVYAGIEVDGDRALIEARQRLEGEGVECLSLSVDELASRRVRSGFWFRDPDGLRLEVVHGL
ADAGTPFRSDIGAGFVTDDEGLGHIVLAVSDLDRAVAFYERVGFAISDFITAPVGPEMVLRIAFLHCNARHHSLALAPL
PGGKRLNHLMLEMTGLDDVLRAHRRSVDRGFVTGGLGRHTNDRMLSFYATTPAGFDVECGFGGCKIEGKPEVREYDAIS
FWGHERSS

A18: Deduced amino acid sequence of extradiol dioxygenase Edo 8 from *Sphingomonas wittichii* RW1.

MSVSNIRYRRLGYLALNVSDLERSRAFYTDVLGLTDDGAGGADRVYLRCSDKHHDLLLVRGDVPGVKRAAWQMENRDAL
EAARAHFAEIGLAPRPVDAAECRDLGIEEAFRISEPTTGVTFEFYATMAELAPFVPSHTDIQRLGHIVLSSTDRPATEA
FLREHMNFRVSDRVDNIVSFMRCFPNPLHHSLGVGGGTRIGLNHVNFMVSNIDDIGRAYHRVKANGVKIVYGPGRHPPS
DSIFLYFLDPDGMTVEYSFGMEEFPEVGAREARLLPAKLESLDSWAAVPDPEFGKHGEIERLPAESAA

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